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SATELLITE DNA IN MAN AND THREE HIGHER PRIMATES

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**Doctorate of Philosophy
University of Edinburgh**

1974

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SUMMARY

Satellite DNA in man and three higher primates

1. A review of the literature shows that satellite DNAs are found in virtually all families of higher organisms and that, for the most part, they are species specific. Work reported in this thesis concerns the isolation and characterisation of satellite DNAs from various primates (including man) and a study of their species specificity.
2. Human satellite DNAs I and III were isolated from human placental DNA and were found to have buoyant densities in neutral CsCl of 1.687 gm/ml and 1.698 gm/ml respectively. Radioactive RNA was made complementary to these fractions and hybridised to human DNA fractionated in cesium salt density gradients and in situ to human metaphase chromosomes. Satellite I was located in the large block of heterochromatin on the distal arm of the Y chromosome, and in the heterochromatin of a C group chromosome, probably chromosome 9. Satellite III hybridised chiefly to the pericentromeric heterochromatin of chromosome 9 and to a lesser extent to the acrocentric chromosomes.
3. Satellite DNA fractions were sought in the DNAs of chimpanzee, orangutan and baboon using CsCl and Ag^+ - and Hg^{++} - Cs_2SO_4 density gradients. In the chimpanzee two satellite fractions were found which had buoyant densities of 1.696 gm/ml and 1.683 gm/ml in neutral CsCl. They were named satellites A and B respectively. Radioactive RNA was made complementary to chimpanzee satellite A and hybridised to chimpanzee DNA, fractionated in cesium salt density gradients and in situ to chimpanzee metaphase chromosomes. Satellite A was located in the pericentromeric heterochromatin of approximately 9 pairs of chromosomes.

4. In the orangutan there was evidence to suggest the presence of a satellite DNA similar to human satellite III and chimpanzee satellite A.

5. In the baboon a satellite DNA with a buoyant density of 1.702 gm/ml was extracted, transcribed and hybridised to baboon DNA fractionated in cesium salt density gradients and in situ to baboon metaphase chromosomes. H was located in the centromeric heterochromatin of all or nearly all of the chromosomes.

6. Cross hybridisations were carried out between the various primate DNAs using complementary satellite RNA and DNA fractionated in cesium salt density gradients or DNA immobilised in situ in metaphase chromosome spreads. Human satellite III and chimpanzee satellite A were found to be homologous fractions and were located in similar positions in the genomes of human, chimpanzee and orangutan. Cross hybridisation did not occur between baboon satellite cRNA and DNA of the three other primates.

REVIEW OF THE LITERATURE

Introduction

When the DNA of various organisms is centrifuged to equilibrium in CsCl density gradients minor components are observed. These components have come to be called satellite DNA and were initially described by Kit (1961, 1962) and Sueoka (1961). Although at first they were operationally defined on the basis of their separation from the bulk of the DNA in isopycnic centrifugation, the discovery that homogeneous kinetically distinct fractions of DNA could be concealed within the "mainband" DNA (Corneo et al, 1970a) led Walker (1971) to define satellite DNA as a native fraction of chromosomal DNA which, after isolation by any method, gives a narrow unimodal band in CsCl because of common properties shared by its sequences. He pointed out that "satellite" was an unfortunate term because it engendered confusion with satellited chromosomes, because analogous DNA fractions had been found concealed within mainband DNA in CsCl gradients, and because it implied a separateness from the bulk of the chromosomal DNA which was entirely dependent on the technique of isolation.

In the eukaryotic genome there is several times as much DNA as is though necessary to code for the requisite number of structural genes (Ohta and Kimura, 1971) and Britten and Davidson (1969) have suggested that some of the excess DNA is involved in organisation and control. Satellite DNAs can be isolated as distinct fractions and provide a means of studying the location and species specificity of a form of this DNA.

Because satellite DNA is a type of reiterated DNA a brief introduction to reiterated sequences will be made before reviewing satellite DNA.

Reiterated DNA

DNA is a molecule which usually consists of two long polymeric strands twisted about each other in the form of a regular double helix. The two strands are joined together by hydrogen bonds between pairs of bases so that adenine is always paired with thymine, and guanine with cytosine. These pairing rules result in a complementary relationship between the sequences of bases so that when strand separation is brought about, the resulting single strands can, under certain conditions, reassociate (Watson, 1965).

Reassociation of DNA can be measured in a number of different ways, each depending on a physical difference between single stranded and double stranded DNA, for instance in their ultraviolet light absorption or their affinity for hydroxyapatite. The renaturation of a pair of complementary DNA strands in solution results from their collision, the frequency of which is concentration dependent. The reaction rate therefore depends upon their concentration and is a measure of the sequence complexity in a given total concentration of DNA. Britten and Kohne (1968) have called this measurement C_{ot} , defined as the product of the DNA concentration in moles/liter times the time taken for renaturation measured in seconds. The DNA of eukaryotes renatures faster than would be expected if each DNA sequence were different and this finding suggested the presence of repetition in the base sequence structure. Britten and Kohne (1968) found repeated sequences in the DNA of all the higher organisms they studied and broadly classified the DNA of eukaryotes as repetitious or as unique sequence DNA. However, this distinction is not absolute and varies somewhat according to the criteria used in the reassociation reaction

and on the molecular weight of the DNA. The higher the temperature and the lower the concentration of salt, the lower the degree of mismatching which will be tolerated in the duplex. Large strands of unhybridised DNA will mechanically disrupt small sections of duplex DNA and so the DNA is usually sheared to uniformly small fragments. The DNA of any particular organism does not normally fall into categories but is viewed as a continuum of species of DNA with different reassociation properties. Table 1 from Britten (1969) lists the observed properties of repeated DNA sequences.

Table 1. Properties of repeated DNA sequences

| | |
|----------------------------|---|
| Occurrence | Observed in all species examined above the fungi |
| Quantity | From 20% to 80% of the total nuclear DNA |
| Frequency | From 50 to 2,000,000 related sequences per family |
| Precision of reassociation | All degrees of thermal stability seen in reassociated repeated DNA |
| Arrangement | Scattered throughout the genome, with extremely repetitious DNA concentrated in the heterochromatin |
| Age | Several million years up to very recent |
| Patterns of occurrence | Patterns of frequency and precision of reassociation vary widely even among vertebrates |
| Expression | RNA complementary to repeated DNA sequences has been observed in every cell type examined |
| Control of expression | Different sets of repeated sequences are transcribed in different tissues and stages of development |

Walker (1969) further divided repetitious DNA into two classes since the most highly repeated sequences did not have the properties shown in Table 1. He therefore divided DNA into three categories: slow, intermediate and fast. Slowly renaturing DNA has a cot value of greater than 10^2 and appears to have only one or a few repeats per genome. It is the least explored of any of the DNAs because of the technical difficulties of identifying individual sequences. It occurs in all species examined, and in DNA reassociation experiments sequence homologies have been detected between closely related species (Britten and Kohne, 1967; McConaughy and McCarthy, 1970). The hybrid and duplex molecules formed from this kind of DNA have a higher thermal stability than those made from more rapidly renaturing fractions (Walker, 1971). Structural genes which are thought to have only one or a few copies per genome are presumed to be found in this fraction.

DNA renaturing at an intermediate rate has a cot value of approximately 1 and has been found in all higher organisms so far examined. It probably contains many families of sequences repeated between 10^2 and 10^4 times, and usually constitutes 10 to 20 per cent of the genome. The intermediate fraction is usually extracted on the basis of its reassociation kinetics although Corneo's human homogeneous mainband fraction (Corneo et al 1970a) may be an example of an intermediate DNA extracted by centrifugation. The characteristics outlined in Table 1 for repetitive DNA are in general those which apply to the intermediate fraction of Walker.

Rapidly renaturing DNA (Cot of less than 1) may not be present in all organisms, is repeated from 10^5 to 10^6 times per genome, and may comprise as much as half of the genome (Bostock et al, 1970),

although in most instances it is under 10% (e.g. mouse, Kit, 1961). The fast fractions of some organisms can be separated from the bulk of the DNA in caesium salt density gradients, and as such are known as satellite DNA. The characteristics of this DNA are discussed in the following section.

Satellite DNA

Most of the properties of satellite DNA are based upon observations made on the mouse. However, data are available for other species and particularly well characterized are satellite fractions in mammalian (guinea pig, rat, calf, monkey, man), amphibian (Xenopus, Plethodon) and invertebrate genomes (Drosophila, Dytiscus, Rhynchosciara, Acheta, Myrmeleotettix, Cancer). The reader is referred to Walker (1971), Coudray et al (1970), Arrighi et al (1970a), and Ingle et al (1973) for summaries of the satellite fractions which have so far been found in the plant and animal kingdoms. Ribosomal cistrons can occasionally be removed as satellite fractions (e.g. Xenopus laevis, Birnstiel et al, 1971). However they will not be considered in detail here as they do not always classify as satellite DNA and constitute an independent area of research (reviewed by Birnstiel et al, 1971).

1. Characteristics of Satellite DNA

a. General properties

Satellite DNA constitutes variable proportions of the genome, from less than 1% in human satellite I DNA (Corneo et al, 1967) to more than 50% of the genome of the kangaroo rat (Hatch & Mazrimas, 1970). By definition satellite DNA separates from the bulk of the DNA in density gradients (Walker, 1971), where it may be found on the light side, as

in the case of mouse (Kit, 1961; 1962), the heavy side, as in guinea pig DNA (Yunis and Yasmin, 1970), or coincident with the mainband as in human satellite IV (Corneo et al, 1972). Satellites coincident with the mainband can be isolated by altering their densities with the chelation of heavy metal ions and subsequent centrifugation in Cs_2SO_4 . The characteristic buoyant density of the satellite is, however, usually stated as that found in neutral CsCl . Except for poly d(A-T) sequences, the lowest density satellite reported is 1.676 gm/ml in the blue crab (Skinner et al, 1970), although a small satellite with buoyant density of 1.669 gm/ml was briefly mentioned in Dros. melanogaster and Dros. virilis (Blumenfeld and Forrest, 1972). The highest density satellite reported is one of density 1.728 gm/ml in the red-backed salamander (MacGregor and Kezer, 1971).

The characteristic buoyant density of a DNA normally reflects its GC content (Schildkraut et al, 1962) but this is not always true for satellite DNAs. In the mouse (Bond et al, 1967; Schildkraut and Maio, 1968) and guinea pig (Corneo et al, 1968c) discrepancies were found when the G+C content as determined by chemical base composition was compared with estimates based on melting temperature and buoyant density. Such discrepancies were not found in the mainband DNA (Schildkraut and Maio, 1968) and may be due to the presence of an unusual base or to a particular structural configuration (Corneo et al, 1968c). Poly d(A-T) is known to manifest a similar peculiarity (Marmur and Doty, 1962). In the chelation of the heavy metal ions Ag^+ and Hg^{++} to DNA, selective binding normally occursⁱⁿ which Ag^+ binds to GC-rich DNA and Hg^{++} binds to AT-rich DNA. Some satellite fractions manifest differences from this predicted behaviour,

discrepancies which have been attributed to the properties of particular nucleotide sequences, or, again, to different structural configurations. Certain of the human and guinea pig satellite fractions chelate either more or less heavy metal than would be expected from their G+C/A+T ratio (Corneo et al, 1968c, 1970a, 1970b, 1971).

b. Strand separation

Strand separation into a heavy (G+T-rich) and a light (A+C-rich) strand takes place when some satellite DNAs are centrifuged in alkaline CsCl (Vinograd et al, 1963). Such strand separation is the property of a reiterated simple sequence DNA, where the base distributions possible in a short segment are limited. For instance, a segment 6 nucleotides long (e.g. guinea pig α satellite, Southern, 1970) could not possibly contain the four bases evenly distributed. Satellites for which alkaline CsCl separation have been demonstrated include the mouse (Flamm et al, 1967; Corneo et al, 1968c), guinea pig (Corneo et al, 1968c, 1970b), calf (Corneo et al, 1970b), man (Corneo et al, 1968a, 1970a, 1971) and the African green monkey (Maio, 1971). Hennig and Walker (1970) failed to find strand separation in the satellite DNAs of two families of rodents, and Yasmineh and Yunis (1971) reported similar results in a calf satellite. It is possible in such cases that the simple sequence has a balanced base composition such that both strands have a similar or identical buoyant density. Alternatively, an exchange of DNA may have occurred between the two strands, and there is evidence in the mouse which suggests that a small proportion of each strand is composed of a short length of the complementary strand (Flamm et al, 1968b).

c. Absorbance Temperature Profiles

Thermal denaturation experiments can indicate the presence of more than one population of molecules if the populations differ in G+C/A+T ratios. Absorbance temperature profiles (melting curves) have been determined for a number of satellite fractions. The native satellite DNAs melt sharply (e.g. mouse satellite DNA, Bond et al, 1967) indicating a high degree of molecular homogeneity (Mandel and Marmur, 1968). However, the melting profile of Dros. melanogaster satellite (Gall et al, 1971) showed three distinct populations of molecules; two sharp melting transitions representative of two populations of satellite DNA sequences, and a gradual transition probably due to contamination with mainband sequences. The so-called "homogeneous mainband" fraction of Corneo et al, 1970a), on the other hand, shows a single melting profile but possibly constitutes two populations of molecules with the same G+C/A+T ratio, namely human satellite III and a fraction of mainband DNA with the same buoyant density (Mitchell, personal communication).

The difference in melting temperature between native and renatured DNAs indicates the amount of mismatching present in the reassociated duplex (Marmur and Doty, 1961) and therefore in the case of satellite DNA, estimates the number of changes which have accumulated in it since its origination. There are few thermal denaturation curves for renatured satellite DNAs (remelts) in the literature. The remelt for mouse indicated that it consisted of a small percentage of poorly matched sequences melting over a broad temperature range, and a majority of well matched sequences giving a sharp thermal transition (Flamm et al, 1967). Similar results were obtained independently for

mouse, guinea pig and human (Corneo et al, 1968c, 1970b, 1968a, 1970a, 1971). Generally, all showed an initial gradual transition of poorly matched regions over a broad temperature range, followed by a sharp thermal transition. Comparisons and meaningful interpretations of published data are difficult because absorbance temperature profiles of satellite fractions have been expressed in various ways; as a percentage increase in hyperchromicity and as direct readings of ultraviolet absorbance on sonicated and unsonicated DNA samples of unspecified molecular weight and in different buffer systems, all of which influence the measurements.

d. Satellite DNAs as repetitive sequences

Although the kinetics and extent of renaturation of purified satellite DNAs have not been studied extensively, it is generally assumed that all satellite fractions are rapidly renaturing. There are several reasons for this. Where cot values have been measured the satellites have been found to reassociate rapidly*. Mouse satellite has a cot of about 5×10^{-3} (Britten and Kohne, 1968), guinea pig satellite II a cot 1/3 that of mouse and guinea pig satellite I approximately 100 x that of mouse (Corneo et al, 1968c, 1970b). Where cot values have not been recorded the DNA has been found to behave as a rapidly renaturing fraction. Evidence of this kind has come from searching for species of rapidly renaturing DNA by buoyant density determinations, as in human satellite DNA (Corneo

* "Rapid" is an arbitrary classification, for example, Walker (1971) says intermediate DNA has a Cot value ranging from 10^{-4} to 1. Eckhardt and Gall (1971) say the satellite DNA in Rhynchosciara hollaenderi "reassociates extremely fast" with a cot of less than 1.

et al, 1968a, 1970a, 1971), and from in situ hybridisation experiments in which the reaction has been limited to rapidly reassociating sequences as in the quail (Brown and Jones, 1972) and Drosophila (Hennig et al, 1970).

Although cot values provide an estimate of sequence reiteration they do not reflect possible sequence divergence. Such divergence will increase cot values and therefore cause overestimates of the length of the repeating sequence. The reassociation rate for guinea pig α satellite suggested that it had a repeat length of 10^5 base pairs (Corneo et al, 1970b). However, Southern (1970) used pyrimidine tract analysis to show that the basic repeating sequence was much shorter, being approximately 6 nucleotides long. He suggested that during evolution point mutations had accumulated in the millions of copies of this sequence, thereby masking the basic repeating unit, and decreasing the renaturation rate. A similar, though less pronounced difference, exists in the case of mouse satellite DNA. Renaturation kinetics suggested it had a repeating unit 300 to 400 base pairs long (Britten and Kohne, 1968), but Southern showed it to be 8 - 13 base pairs long. Estimates of length of repeating sequence based on the kinetics of renaturation are therefore bound to be overestimates. As the degree of mismatching increases, the apparent sequence length calculated from reassociation kinetics also increases.

In pyrimidine tract analysis diphenylamine is used to degrade the purines and so liberate tracts of pyrimidine which can be used to deduce the DNA sequence. However, the method suffers not only from being laborious, but also that it can only be applied to those DNAs which are capable of undergoing complementary strand separation.

Thus it is unlikely to be used frequently to discover the actual repeat length of particular satellite DNAs. Aside from the restriction endonucleases it is unfortunate that there are no enzymes for the specific cleavage of DNA such as the various ribonucleases used to cleave RNA. It is therefore probable that estimates of repeat length based on renaturation rates will continue to be cited in the knowledge that they are overestimates.

e. Species specificity of satellite DNAs

The overall base compositions of satellite DNAs from different species differ as widely as do the relative proportions of satellite DNA present. Walker (1971) pointed out that no two species had been shown to share the same kind of satellite DNA, although the presence in one species of a few satellite sequences similar to those found in a related species could not be ruled out.

The question of species specificity of satellite DNA was initially introduced in the literature in relation to mouse satellite sequences. McLaren and Walker (1966) hybridised various fractions of mouse DNA with DNA from other rodents. They found no similarity in the mouse satellite fraction but did demonstrate a similarity in fractions other than satellite. Walker (1968); Walker et al (1969) and Flamm et al (1969b) looked at the density gradient patterns of DNA from several species of rodents, Rattus norvegicus, Cavia porcellus, Apodemus sylvaticus, Peromyscus maniculatus and Peromyscus polionotus and found that they had satellite sequences which were different both from each other and from Mus musculus. The results of cross-hybridisation between single-stranded mouse satellite DNA and the DNA from these other five rodents indicated that they had less than 1 in 10^6

sequences in common. Hennig and Walker (1970) reported on 16 different species, subspecies and geographically isolated populations within two rodent families and found different amounts and different densities even in very closely related animals.

Evidence to support the species specificity of satellite DNA has been advanced from other sources. In 1969 Gall et al, showed that three species of Dytiscus beetles had different satellites. Gall (1970) later showed that the satellite in each of 12 species of Dytiscus was species specific with regard to the others. Satellite fractions of different buoyant densities and different amounts have been demonstrated in several species of Drosophila (Laird and McCarthy, 1968; Etingh, 1970; Gall et al, 1971). Hennig et al (1970) looked at the satellites in three sibling species of Drosophila and found that the majority were species specific, although a heavy satellite in Dros. neohydei was found by in situ hybridisation to be common to Dros. hydei and Dros. pseudoneohydei.

The DNA of twelve species of Dipodomys exhibited a unique pattern in CsCl gradients (Mazrimas and Hatch, 1972). In the grasshopper (Myrmeleotettix maculatus) the presence or absence of satellite DNA is dependent on the presence or absence of B chromosomes (Gibson and Hewitt, 1970). In populations collected from various ecological niches, there was no homology between the cRNA synthesized from B chromosome DNA of one population with the B chromosome DNA of another population (Gibson and Hewitt, 1972). This was evidence not only for species specificity, but for population specificity within a species.

However, there is evidence that not all satellite fractions are species specific. A satellite DNA consisting almost entirely of

poly d(A-T) sequences was originally found in varying proportions in various species of crab (Sueoka, 1961; Sueoka and Cheng, 1962; Smith, 1963, 1964). It was shown to be located within the nucleus (Astell et al, 1969) and to be present in similar proportions in different tissues (Cheng and Sueoka, 1964). Whether such a satellite can be considered species specific depends on the criteria accepted for such specificity. Klett et al (1969) found poly d(A-T) distributed in a number of families in the superfamily Brachyrrhyncha, to which Cancer belongs. From the buoyant densities and thermal denaturation temperatures he deduced that there were small but significant differences in the structure of the satellite in the different species. Skinner (1967) found the poly d(A-T) from Geocarinus lateralis and Cancer pagurus consisted chiefly of alternating thymidylate and adenylate residues, but that a significant proportion consisted of adjacent thymidylate residues and differed between the two species. In four species of crab Skinner et al (1970) found four satellites of the same buoyant density (1.677 gm/ml); three of these were poly d(A-T) and one constituted a satellite with approximately 27% G+C. The need for very rigorous testing was further emphasized by the case of the AT-rich satellites in the DNAs of two distantly related species of Crustacea, a freshwater crayfish and a marine crab (Graham and Skinner, 1973). The satellites had identical buoyant densities in neutral CsCl and in alkaline CsCl each formed two distinct bands of very similar densities, but the Tms differed markedly showing that they were different.

Swartz et al (1962) found poly d(A-T) in Cancer borealis was 93% alternating A+T residues, 3% G+C, and the remainder consisted

of A+T in sequences other than alternating copolymer. It is possible that species specificity exists in crab poly d(A-T) in the small but variable proportion of the satellite which is not alternating copolymer, but at the level of cross hybridisation it is not species specific. From nearest neighbour analysis Fansler et al (1970) found that Dros. melanogaster poly d(A-T) had a distinctive structure differing from other poly d(A-T)s and Blumenfeld and Forrest (1971) suggested the arrangement of dA and dT in the copolymer was species specific.

Graham and Skinner (1970) cross hybridised the cRNA of a GC rich satellite from Gecarinus lateralis to the DNA of various other Crustacea and found as much as 20% homology. Approximately half of the Gecarinus repetitious mainband DNA sequences were found to be represented in the DNA of the other true crabs (Graham and Skinner, 1973) and the heterologous hybrids formed indicated that there had been little evolutionary divergence of the conserved sequences. 5 - 10% homology to the G+C-rich satellite was observed. The thermal transitions of these hybrids indicated that the satellite-related sequences had apparently undergone more divergence than the conserved repetitious sequences present in mainband DNA.

Several species from the genus Mus contain satellite DNAs able to cross hybridise with the Mus musculus satellite. In four species of the genus at least five related satellite DNAs exist which have been concluded to be a family of related sequences (Sutton and McCallum, 1972).

2. Location in the Genome

a. Extrachromosomal

When the presence of satellites was first noticed in higher organisms,

the danger of confusing them with extranuclear DNA was not appreciated. In unicellular plants and animals of lower species (Gibor and Granick, 1964; Corneo et al, 1966) and in a few plants of higher species (Chun et al, 1963) the satellite DNAs are located in cytoplasmic organelles, the mitochondria and chloroplasts. Among higher animals, satellite fractions in the chick, duck and pigeon are due to mitochondrial DNA (Borst and Ruttenberg, 1966; Borst et al, 1967) while in the calf, guinea pig, rat and mouse mitochondrial DNA has been shown to have a buoyant density identical or similar to mainband DNA (Flamm et al, 1966b; Corneo et al, 1966; Borst and Ruttenberg, 1966). Bond et al (1967) purified both mouse satellite DNA and mouse mitochondrial DNA and showed that they banded separately. Corneo et al (1966) concluded that the density of mitochondrial DNA was usually different from the density of the nuclear DNA in simple unicellular organisms (moulds, yeast, algae, protozoa) while it was apparently the same as nuclear DNA in higher mammalian species.

b. Nuclear

Mouse satellite DNA constitutes about 10% of the genome regardless of the tissue from which the DNA has been prepared, and is found in the same proportions in cultured cell lines (Kit, 1961, 1962; Chun and Littlefield, 1963; Schildkraut and Maio, 1968). It is found in germ-free mice and in males and females to the same extent (Waring and Britten, 1966). It is exclusively intranuclear in origin (Flamm et al, 1966b) and is found in the same amount in metaphase and interphase nuclei (Maio and Schildkraut, 1967). When mouse metaphase chromosomes were separated according to size in sucrose gradients, the proportion of satellite DNA relative to total DNA was

approximately the same in all fractions (Schildkraut and Maio, 1968; Maio and Schildkraut, 1969). This was later confirmed with in situ hybridisation by Jones (1970) and Pardue and Gall (1970) who showed mouse satellite cRNA was located on the centromeric heterochromatin of all chromosomes except the Y.

c. Association with heterochromatin

Before discussing the relationship which exists between heterochromatin and satellite DNA, it may be beneficial to define several relevant terms. The distinction between heterochromatin and euchromatin is usually made on a cytological basis, heterochromatin stains darkly while euchromatin stains lightly or not at all. Heterochromatin tends to occur in similar regions in the different chromosomes of a species, most often immediately next to the centromeres, at the ends of chromosomes, and in the vicinity of the nucleolus organiser. It is frequently a large constituent of sex chromosomes and the extra or supernumerary chromosomes which occur in some species (Brown, 1966).

A further distinction is made between two kinds of heterochromatin. In constitutive heterochromatin the DNA is thought to be genetically inactive in all tissues and at all times, that is, it is thought to contain few if any major genes, whereas in facultative heterochromatin the native DNA is thought to contain potentially transcriptionally active sequences subject to controls operating in different tissues or different stages of development.

Although both types of heterochromatin replicate late in the cell cycle there is evidence that they replicate at different times (Huang, 1971; Schmidt and Leppert, 1969). Constitutively heterochromatic segments associate with each other so that a lower number of chromocentres

are seen per interphase nucleus than the number of heterochromatic regions in the chromosome complement (Jones, 1970; Natarajan and Ahnstrom, 1970; Pardue and Gall, 1970), whereas facultative heterochromatin forms individual chromocentres such as Barr bodies (Barr, 1965) and sex vesicles (Pardue and Gall, 1970).

The relationship of satellite DNA to constitutive heterochromatin has been established in several organisms in different ways. Yasmineh and Yunis (1969, 1970) fractionated mouse nuclei into heterochromatin and euchromatin fractions and found a seven-fold increase in satellite DNA in the heterochromatin fraction. Matoccia and Comings (1971) found a two- to three-fold enrichment. A similar enhancement of satellite DNA was observed in the heterochromatin preparations of guinea pig (Yunis and Yasmineh, 1970), calf (Yasmineh and Yunis, 1971), kangaroo rat (Mazrimas and Hatch, 1970), human (Corneo et al., 1971) and crab (Duerkson and McCarthy, 1971). Usually male tissue was examined thereby reducing facultative heterochromatin in the preparations. An enrichment in satellite DNA was found in all heterochromatin preparations in all species examined except in the African green monkey (Maio, 1971).

By the technique of in situ hybridisation Jones (1970) and Pardue and Gall (1970) showed that mouse satellite was preferentially located in chromocentres and nucleolar regions at interphase. At metaphase the centromeric heterochromatin of all chromosomes except the Y bound mouse satellite DNA. Human satellite sequences were located in centromeric heterochromatin of some chromosomes and in heterochromatic regions at interphase (Jones and Corneo, 1971; Jones et al., 1973). In the amphibian Plethodon c. cinereus a heavy satellite was located in the

centromeric heterochromatin of all chromosomes (MacGregor and Kezer, 1971). The relationship between heterochromatin and satellite sequences has been shown cytologically in various insect species, in Rhynchosciara hollaenderi (Pardue et al, 1970; Eckhardt and Gall, 1971), Dros. melanogaster and Dros. hydei (Pardue et al, 1970; Hennig et al, 1970). In the quail a heavy satellite is situated chiefly in the microchromosomes (Brown and Jones, 1972) which are thought to be heterochromatic and which form a chromocentre at interphase (Comings and Mattocia, 1970a).

Evidence has accumulated suggesting that satellite DNA replicates late, with the heterochromatin fraction. Tobia et al (1970) and Flamm et al (1971) showed that mouse satellite DNA replicated in the second half of the S period, and Bostock and Prescott (1971) restricted this time to the third quarter of S. They found little or no satellite replication in the fourth quarter but observed a gradual change as S progressed with DNA of heavier than average buoyant density replicating first. In the kangaroo rat Bostock et al (1972) found that the satellites replicated in the second half of S. Although Comings and Mattocia (1970b) indicated that mouse satellite replicated with the euchromatin fraction of the genome, the weight of evidence that both heterochromatin and satellite DNA replicate late in S has been taken to indicate a relationship between the two.

Smith (1970) observed that when host cell DNA synthesis in mouse kidney cells was switched on with polyoma virus infection, all the light satellite DNA replicated before the remainder of the cell DNA. He suggested that by upsetting the role of satellite DNA in the cell an oncogenic virus might attack the ordered sequence of events in

usual cell division. However, these results were subsequently questioned by Hatfield and Walker (1973) who were unable to reproduce Smith's findings. They concluded that the time of replication of satellite DNA in cultures infected with polyoma virus was similar to that in uninfected cells.

d. Nucleolar association

There is evidence from studies on various animals that a relationship exists between satellite DNA and the nucleolus. In the mouse Schildkraut and Maio (1968) found a three- to four-fold enrichment of the satellite fraction in nucleolar DNA preparations (although Bond et al [1967] found the proportion of mouse satellite in their nucleolar preparations was the same as that found in the total nuclear DNA). In the mouse the secondary constrictions (nucleolus organisers) are located near the centromeres of several chromosome pairs (Levan et al, 1962) and therefore adjacent to the centromeric heterochromatin. The association of satellite DNA with nucleoli might therefore be trivial. However Schildkraut and Maio (1968) found that treating the nucleoli with 2 M NaCl removed relatively more mainband than satellite DNA, and suggested that satellite DNA was more tightly bound to the nucleolus. This observation was corroborated by Mattoccia and Comings (1971) who extracted DNA containing 55% satellite from mouse nucleoli treated with 2 M NaCl. The unequal distribution of satellite and mainband DNA in DNA from nucleolar preparations suggested that satellite DNA might be limited to chromosomes of a specific type or size, specifically those containing nucleolus organiser regions, but this was not found to be the case (Schildkraut and Maio, 1968; Maio and Schildkraut, 1969). In situ hybridisation results of Jones (1970)

showed that mouse satellite rRNA was concentrated in the vicinity of nucleoli in interphase cells.

A similar enrichment of satellite DNA was found in nucleolar preparations from the field vole (Schildkraut and Maio, 1968), the kangaroo rat (Mazrimas and Hatch, 1970), the African green monkey (Maio, 1971) and man (Schildkraut and Maio, 1969).

3. Function of Satellite DNA

a. The lack of transcriptional activity

Ten years after its discovery, the function of satellite DNA is still unknown. In the mouse, satellite DNA does not appear to be transcribed (Flamm et al, 1969; Walker, 1971) although Harel et al (1968) reported that rapidly labelled RNA bound to one mouse satellite strand to a greater degree than it did to mainband DNA. Walker (1971) pointed out that from the low hybridisation values they obtained for a highly reiterated fraction it was possible the RNA bound to a minor component of the satellite fraction. Cohen et al (1973) found that mouse L cells contained RNA capable of hybridising with satellite DNA. Their method of satellite preparation depended on the rapid reannealing rate of the satellite as well as selective binding of Ag^+ ion. It is possible that they extracted some rapidly renaturing non-satellite DNA. They found 4% hybridisation of RNA to pure satellite DNA on filters.

In Rhynchosciara hollaenderi the chromosome puff regions do not hybridise with satellite DNA indicating that it is not the satellite that is being transcribed (Eckhardt and Gall, 1971).

The known primary structure of the basic repeating sequence in satellite DNA (Southern, 1970) seems to rule out the possibility that the DNA codes for a protein. Of the possible reading frames for the two strands of the guinea pig α satellite two would give alternating

nonsense codons and the others repeating dipeptides.

Greenberg and Uhr (1967) reported that 16S RNA bound four times better to satellite DNA than to mainband DNA in the mouse. However, since rDNA has been found in many eukaryotes to band at a density greater than that of the bulk of the DNA (Birnstiel et al, 1971) it is unlikely that the light density mouse satellite contains ribosomal cistrons. In certain species however, Xenopus laevis (Birnstiel et al, 1968), Dytiscus beetles (Gall et al, 1969; Gall, 1970) Acheta (Lima-de-Faria et al, 1969) and the sea urchin (Stafford and Guild, 1969) a proportion of the satellite DNA has been found to code for ribosomal cistrons. Except for this special instance of transcription of satellite DNA into ribosomal RNA, no well authenticated transcription of a satellite fraction has been demonstrated.

b. Satellite amplification in polytene chromosomes

In the giant trophoblast cells of the mouse placenta, in which each nucleus has several hundred times the haploid amount of DNA, satellite DNA and mainband DNA are found in normal proportions (Sherman et al, 1972). Disproportionate accumulation is found in Dros. melanogaster and Dros. virilis where the satellite DNA is under-represented in polytene tissues (Gall et al, 1971). A similar result has been reported by Hennig and Meer (1971) in Dros. hydei. In Dros. virilis of the three satellites studied, two were under-replicated in adult tissue and one was not (Blumenfeld and Forrest, 1972).

The significance of differential replication of satellite DNA in the genome of certain cells is unknown.

c. Satellite DNA as spacer sequences

In the organisms in which the ribosomal cistrons occur as a satellite fraction, in no instance does ribosomal DNA account for the entire fraction. In Xenopus laevis approximately 40% of the satellite codes for ribosomal cistrons, the remainder is accounted for by spacer sequences (Birnstiel et al, 1968). In Dytiscus beetles less than 1% of the satellite is ribosomal cistrons, the rest being presumed to be ribosomal spacer DNA (Gall et al, 1969; Gall, 1970). Gall (1970) suggested that satellite DNA could, in general, represent spacer sequences for as yet undetected amplified genes, though even if this is true, ignorance as to the function of spacer sequences still prevents an understanding of the role of satellite DNA.

Walker (1971) discussed the parallels between satellite DNA and ribosomal spacer DNA in Xenopus. He pointed out that the spacer varied in length in the amplified ribosomal cistrons of Xenopus by as much as a factor of 10 (Miller and Beatty, 1969) and that the repeating units of satellite DNA also vary in size (Britten and Kohne, 1968; Southern, 1970), also, that the lack of homology between the spacer DNA of Xenopus laevis and Xenopus mulleri, species which will interbreed in the laboratory (Brown et al, 1972) is analogous to the lack of homology in satellite DNAs between even closely related species.

d. An evolutionary function

A relationship between satellite DNA and the evolution of the kangaroo rat was suggested by Mazrimas and Hatch (1972) who looked at 12 of 22 species of kangaroo rat where the amounts of satellite material ranged from barely detectable to more than half the total

nuclear DNA. They found the proportion of satellite to mainband DNA correlated directly with the number of recognised subspecies and with the chromosomal fundamental number, and inversely with the extent of evolutionary specialisation. They suggested that this was evidence that satellite DNA was one of the mechanisms that has evolved in higher organisms to promote genetic flexibility.

A similar situation exists in grasshoppers where the presence of satellite DNA is dependent on the presence of supernumerary or B group chromosomes. In populations with B chromosomes satellite DNA is present, in populations without B chromosomes satellite DNA is absent (Gibson and Hewitt, 1970, 1972). The frequency of individuals with B chromosomes decreases in less favourable environments so that populations in very stringent environmental conditions have none. They therefore suggested that B chromosomes conferred variability which had selective advantages.

MATERIALS

a. Tissues

Human placental tissue was obtained for the preparation of human DNA from the Simpson Memorial Maternity Pavilion in Edinburgh. The chimpanzee (Pan troglodytes) liver was a gift from Dr Martin Bobrow, Oxford, the baboon (Papio cyncephalus) liver was a gift from Dr A.K. Sim, Inveresk Research International, Scotland, and the orangutan (Pongo pygmaeus) was a gift from Dr K. Benirschke, San Diego, California.

Fresh human blood was obtained from donors in the laboratory, and samples of baboon blood were provided by Dr Sim. Slides of chimpanzee and other primates were obtained from Dr Bobrow.

b. Culture medium

Eagles MEM (Hanks based plus non essential amino acids) was obtained from Burroughs Wellcome. To this medium was added 0.11% w/v sodium bicarbonate, 10% fetal bovine serum from Biocult Ltd., Glasgow, and antibiotics: 0.13 mg/ml streptomycin and 60 µg/ml penicillin, both from Glaxo Laboratories, Greenford

c. Scintillation fluid

The Toluene based scintillation fluid contained 2.5 liters toluene, 12.5 gm PPO (2,5 diphenyloxazole, scintillation grade) and 0.75 gm POPOP (1,4 bis 2-(5 phenyloxazolyl)-benzene, scintillation grade).

Unless otherwise indicated, all chemicals were obtained from British Drug Houses (BDH).

METHODS

1. Preparation of Chromosome spreads

a. Fibroblast, HeLa and L-cell technique

1. Cells were grown to half confluence in 9 cm Petri dishes (Sterilin, Flow Laboratories, Ayrshire) in 10 mls culture medium.
2. Colcemid (Ciba) was made up 1 mg in 100 mls Dulbecco A (Oxoid) and added at 0.4 mls/10 ml dish for the last 4-6 hours of culture.
3. Culture medium was carefully pipetted off and the cells washed once, very gently in Dulbecco A.
4. Trypsin (Difco, Detroit, Michigan) was made up 0.25% in Dulbecco A and sterilized through Millipore filters (HAWP, 0.22 μ pore size). Approximately 5 mls was added per culture dish. The dish was gently agitated until cells began to "lift". The trypsin was gently swirled into the next culture dish. One 5 ml portion of trypsin was used on 3-4 Petri dishes in this way before being poured into a 10 ml conical centrifuge tube.
5. The cells were spun on an MSE bench centrifuge at 2 (\sim 1400 rpm) for 3 minutes.
6. The supernatant was poured off and the cells resuspended in the residual supernatant.
7. Approximately 3-4 mls 0.075 M KCl was added for 5-8 minutes and the cells again spun at 2 for 3 minutes.
8. The supernatant was poured off and the cells resuspended in the residual supernatant.
9. Freshly prepared fixative (methanol: glacial acetic acid, 1:1) was carefully added dropwise down the side of the tube with constant agitation and left for 5 minutes.

10. Three changes of fixative were carried out and the cells resuspended in a small amount of fixative, sufficient to give plating at the desired concentration.

11. One drop of cell suspension was dropped onto a slide (previously cleaned in chromic acid and rinsed in 1:1 alcohol:ether) and either air- or flame-dried. One slide from each preparation was stained to check the quality of the chromosome spreads. Good quality chromosomes were stored in vacuo until required.

b. Whole Blood Culture Technique

1. Peripheral blood was drawn by venipuncture into a disposable plastic syringe and immediately transferred to a plastic lithium heparin bottle (Stayne Laboratories, High Wycombe) and thoroughly mixed. If heparin bottles were not available, heparin (1000 units/ml, Boots, Nottingham) was added to the sample at a concentration of 0.1 ml per 5 ml blood.

2. Universal bottles (10 ml) were prepared containing 4 ml culture medium (see Materials) and 0.05 ml phytohaemagglutinin (Burroughs Wellcome) made up as directed on the phial.

3. 0.4 ml heparinized blood was added to each bottle and the culture incubated at 37°C for 2-3 days.

4. 0.5 ml of a 0.02% solution colcemid (Ciba) made up in Dulbecco A (Oxoid) was added for the last 3 hours of incubation.

5. The cultures were gently shaken, transferred to 10 ml conical centrifuge tubes and spun down on an MSE bench centrifuge at 5 for 5 minutes.

6. The supernatant was discarded. The button of cells was re-suspended in the residual supernatant and 5-10 mls of 0.075 M KCl was

added for 5-8 minutes.

7. The culture was centrifuged at 5 for 5 minutes and the supernatant discarded. The button of cells was resuspended in the residual supernatant before fixative was added.

8. Freshly prepared fixative (3 parts methyl alcohol : 1 part glacial acetic acid) was added very slowly dropwise down the side of the tube and with constant stirring.

9. The cells were given 3 changes of fixative and finally resuspended in sufficient fixative to allow plating at the desired concentration.

10. One drop of cells in suspension was dropped onto a clean slide and left to dry.

11. A slide from each preparation was stained to assess the quality of the chromosome spreads. Good quality chromosomes were stored in vacuo until required.

2a. Preparation of DNA from whole tissues

A modification of the methods of Marmur (1961) and Kirby (1957) was used in the preparation of DNA. In order to avoid nuclease degradation of the DNA the tissues used were either fresh or fresh-frozen, and all steps until the addition of SLS were carried out at 0°C. To avoid shearing the DNA molecules, the preparation was treated with gentle shaking and pipettes were used inverted.

1. The tissues were minced and placed directly in saline EDTA at 0°C (EDTA chelates metal ions necessary for nuclease activity) and washed several times to remove blood.

2. The connective tissue, particularly of placenta, was removed. The tissue was then minced with scissors and homogenized in a loose-fitting glass teflon homogenizer (approximately 5 ml saline EDTA to each gram of tissue).

2. SLS was slowly added to a final concentration of 2 gm percent, and the mixture was incubated at 60°C for 10 minutes (SLS lyses the cells, inhibits DNase, and dissociates DNA from protein). If the aliquot was not too viscous after incubation, the remainder of the homogenate was treated similarly; however if a gel formed, then the homogenate was diluted with saline EDTA before the addition of SLS.

4. Sodium perchlorate (5M) was added to a final concentration of 1 M.

5. $\frac{1}{10}$ of a volume of saturated Tris pH8.5 was then added and mixed. (The high salt concentration in steps 4) and 5) dissociates the nucleoprotein complex).

6. An equal volume of chloroform-isoamyl alcohol (24+1, v/v): phenol, 1:1, was added and the solution agitated for 1/2 hour.

(Both further dissociated the DNA from protein).

7. The shaken material was transferred to centrifuge tubes or centrifuge bottles and spun at 10K rpm (~12,000 G) for 10-20 minutes in the Sorval superspeed refrigerated centrifuge (RC2 B) at 4°C.

8. The top aqueous layer was withdrawn with an inverted pipette and transferred to a beaker.

9. Two volumes of absolute ethanol were carefully overlaid on the DNA solution, and a glass spooling rod was used to wind the DNA molecules out of solution. (Ethanol precipitates nucleic acids from aqueous solution). The crude DNA was dehydrated through 80%, 90% and absolute ethanol, air dried and dissolved in 0.1 x SSC.

10. After the crude DNA was dissolved in 0.1 x SSC, 1/10 volume of 10 x SSC was added to the solution and solid NaCl was added to make the solution 1 M in NaCl.

11(a). RNase (Sigma) was made up in a solution of 2% Na acetate pH5.0 at a concentration of 2mg/ml, heated to 100°C for 5 minutes (to remove DNase), chilled to room temperature and added to the DNA solution to a final concentration of 50 µg RNase/ml of crude DNA.

11(b). Amylase (Sigma) was added to a final concentration of 200 µg/ml at the same time as the RNase. Amylasing was usually carried out, but routinely so when the starting tissue was liver.

11(c). The DNA solution plus enzymes was incubated at 37°C for three hours.

12. Protease (Sigma) was dissolved in saline EDTA at a concentration of 10 mg/ml and added to the solution to a final concentration of 400 ug/ml. The solution was then incubated for 3 - 6 hours at 37°C or overnight at room temperature.

13. The solution was shaken with chloroform-isoamyl alcohol: phenol, 1:1, and centrifuged as in step 6. The aqueous layer containing DNA was removed. This deproteinizing step was repeated until no protein interface was observed between the two layers.

14. Acetate EDTA (3 M Na acetate, 0.001 M EDTA), pH7.0 was added (1/10 volume) and mixed. Isopropanol (0.56 volume) was carefully overlaid on the mixture and the DNA spooled out of solution. (Spooling through isopropanol selectively removes DNA molecules, leaving contaminating RNA in solution).

15. The preparation was dehydrated through 80%, 90% and absolute ethanol, air dried, and dissolved in the desired solvent (e.g. 0.1 x SSC or 0.1 M Na_2SO_4).

16. Care was taken to ensure that all DNA was dissolved before its concentration was determined spectrophotometrically. Final readings at 260 nm were made on serial dilutions for accuracy of estimation.

17. Pure DNA of known concentration was stored at -20°C . To prevent shearing and degradation caused by repeated freezing and thawing, aliquots of DNA in useful amounts were frozen individually.

2b. Preparation of DNA from tissue culture cells

1. Cells cultured to maximum density in Petri dishes were thrice washed with Dulbecco A to remove all traces of culture medium (which contained trypsin inhibitor).

2. Trypsin was made up (0.25%) in Dulbecco A and sterilized through Millipore filters (HAWP, 0.22 μ pore size). It was added at approximately 5 mls per 10 ml Petri dish and incubated at 37°C until the cells were detached from the plate. The cells were then scraped off the dish with a rubber "policeman". One 5ml portion of trypsin was used in this way on 3-4 Petri dishes before being poured into a conical centrifuge tube.

3. The cells were pelleted in an MSE bench centrifuge at 5 (approx. 2300 rpm) for 5 minutes.

4. The supernatant was removed and the cells carefully resuspended in the residual supernatant.

5. Protease (Sigma) was made up at a concentration of 200 ug/ml in 2 x SSC, digested at 37°C for 15-30 minutes, and adjusted to 0.1% with SLS. This solution was added (approximately 3-4 mls per dish of cells) and incubated at 37°C for 2 hours.

6. Solid CsCl was then added at a concentration of 1.26 gm/ml, and 5 ml aliquots of the solution were put in 10 ml MSE centrifuge tubes and centrifuged for 40 hours at 40 K rpm in a 10 x 10 angle rotor in the MSE 50 ultracentrifuge.

7. After centrifuging to equilibrium, 10 drop fractions were collected from a hole pierced in the bottom of the centrifuge tube.

8. The fractions were diluted with 0.5 ml of 0.1 x SSC and read at 260 nm. The fractions containing the DNA peak were pooled and either a) pelleted for 18 hours at 30 K in the 3 x 20 swing out rotor of the MSE 50 ultracentrifuge, then redissolved in a small volume of 0.1 x SSC or b) dialyzed against 0.1 x SSC, concentrated by evaporation, and redialyzed into 0.1 x SSC.

3a. Preparative Fractionation of DNA in Neutral CsCl

The method of isopycnic centrifugation was reviewed by Flamm et al, (1966a, 1969a).

1. To DNA dissolved in 0.1 x SSC at an appropriate concentration (usually not more than 50 ug/ml) was added solid CsCl at a concentration of 1.26 gm/ml, giving an initial buoyant density of 1.700 - 1.720 gm/ml as judged from the refractive index.

2. Five ml/tube were then placed in 10 ml MSE polypropylene centrifuge tubes, overlaid with paraffin oil and centrifuged in an MSE 50 ultracentrifuge for 40 hours at 44 K rpm and 20°C.

3. The centrifuge was decelerated without braking. A hole was pierced in the bottom of the tube and 6 - 10 drop fractions were collected (depending on the desired resolution of the gradient).

4. Fractions were diluted with 0.5 ml 0.1 x SSC and their absorbances at 260 nm were serially determined in a Unicam Sp 800 spectrophotometer and plotted on graph paper.

3b. Preparative Fractionation of DNA in Alkaline CsCl

1. To 15 ug of DNA in 0.8 ml 0.1 x SSC was added 0.025 ml of 1 N NaOH. CsCl was added to a final density of 1.755 - 1.770 gm/ml, as judged from the refractive index and the preparation was centrifuged at 40 K rpm for 40 hrs at 20°C.

2. The gradient was dripped, diluted and read in the same way as for neutral CsCl centrifugation.

3. The samples were dialyzed to remove NaOH.

3c. Preparative fractionation of DNA in Ag^+ - Cs_2SO_4

Silver and mercury ions are among the few cations which form complexes with the nucleic acids by interaction with purine and/or pyrimidine bases, rather than by interaction with the phosphates. Cupric ion can interact with either the phosphate groups or the nucleoside bases, depending upon the ionic strength and other conditions, whereas many other divalent cations, such as Mn^{++} , Mg^{++} and Co^{++} bind primarily to the phosphates (Jensen and Davidson, 1966). Silver and mercury are probably bound between two nitrogens of complementary base pairs. The silver ion is bound more strongly by GC-rich DNA's than by AT-rich ones and the converse is true for mercury ion (Nandi *et al.*, 1965; Jensen and Davidson, 1966), but exceptions to this rule occur

(Jensen and Davidson, 1966; Corneo et al, 1968c; 1970b). The formation of complexes is dependent on the pH and on the molar ratio of metal ion to DNA-P used. The selective binding reaction can be used to create large buoyant density differences between single- and double-stranded DNA or between DNAs with different base compositions. The binding is reversible on addition of a sufficiently strong complexing agent for the metal ion, and the DNA can be recovered in its original form with no deterioration in its structure or biological activity (Jensen and Davidson, 1966).

1. Purified DNA was dissolved in 0.1 M Na_2SO_4 and then dialyzed into 0.1 M Na_2SO_4 to remove all Cl^- ions which would interfere with the chelation of metal ions to DNA. This was routinely used for centrifugation at a final concentration of 70 $\mu\text{g/ml}$.

2. A solution of 0.1 M borate buffer pH9.2 was added to give a final concentration of 0.005 M with respect to the borate ion. A 10^{-3} M solution of AgClO_4 was added to give varying molar ratios of Ag^+ to DNA-P. Molar ratios of 0.15 to 0.35 were used.

3. A saturated solution of Cs_2SO_4 in distilled water ($\rho=1.930$ gm/ml) (Merck, Suprapur Cs_2SO_4 obtained from Anderman and Co. Ltd, London) was added to give the required initial buoyant density as judged from the refractive index measurements. For instance, a refractive index of $7^\circ 5-15'$ was used with R_F of 0.15, and $7^\circ 30-40'$ with R_F of 0.35. In general, an increase in R_F of 0.05 led to an increase in initial buoyant density of 0.012 gm/ml and was achieved by increasing the amount of saturated Cs_2SO_4 added per ml of gradient by 0.013 ml.

4. Preparative centrifugation was carried out in volumes of 20 ml/tube in the 8 x 50 fixed angle rotor of the MSE 50 ultracentrifuge. The preparation was spun at 30 K rpm, 20°C for 96 hours.

5. The centrifuge was decelerated without braking. A hole was pierced in the bottom of the tube and 6 - 10 drop fractions were collected and diluted with 0.5 ml 0.1 M Na_2SO_4 . Their absorbance at 260 nm was determined in the Unicam SP 800 spectrophotometer and the readings were plotted. In some gradients, DNA from individual fractions was analytically centrifuged in neutral CsCl together with marker DNA in order to plot the buoyant density of DNA across the gradient. Ag^+ was first removed as described in step 6.

6. The fractions pertaining to the region(s) of satellite DNA were pooled and extensively dialyzed in 5 M NaCl plus 0.01 M Tris HCl pH7.0 to remove the metal ion, and then dialyzed in 0.1 x SSC.

7. When further purification of the satellite was required, the satellite DNA in 0.1 x SSC was concentrated by evaporation to approximately 30 ug/ml. CsCl (Anal-R) was added to give a final density of 1.700 - 1.720 gm/ml as judged from the refractive index and the solution centrifuged in the 10 x 10 fixed angle rotor of the MSE 50 ultracentrifuge for 40 hours at 42 K rpm and 20°C.

8. Fractions were collected, diluted with 0.5 ml 0.1 x SSC, their absorbance at 260 nm determined in the Unicam SP800 spectrophotometer and the results plotted.

9. After the final purification, the satellite DNA was concentrated by evaporation and dialyzed into the desired solvent.

It was observed that the addition of varying amounts of Ag^+ or Hg^{++} to the same concentrations of DNA in a Cs_2SO_4 gradient produced bands of total DNA of differing widths. It is possible that the

amounts of heavy metal ion chelated under different conditions cause the bands to spread over varying density ranges. The Ag^+ - or $\text{Hg}^{++}\text{-Cs}_2\text{SO}_4$ method for the extraction of the satellites is not always reproducible. In the human genome, for instance, 70 ug/ml of DNA and a molar ratio of $\text{Ag}^+:\text{DNA-P}$ of 0.20 should, according to Corneo et al (1971), produce three human satellites, II, III and I. This was observed on several occasions but it was not a standard result. More often a profile was obtained which showed only satellites III and I (Figure 2a). The chimpanzee $\text{Hg}^{++}\text{-Cs}_2\text{SO}_4$ results presented in the thesis (Figure 19) differ slightly from the result published (Prosser et al, 1973). The baboon results, too, were found to be variable. The microdensitometer tracings reproduced were those which were most often observed, but the light satellite in the $\text{Hg}^{++}\text{-Cs}_2\text{SO}_4$ gradient (Figure 36) was occasionally absent.

It is not clear why this variability occurs. It may depend on the purity of the DNA or on the method of preparation employed. It has been found that the phenol method of making DNA selectively destroys AT-rich regions (Skinner & Triplet, 1967) and this could be responsible for the variations in results when DNAs of different batches were used. The size of the tubes and the volume of solution centrifuged in them was indicated for each preparation since it was sometimes found that different OD profiles were obtained for gradients centrifuged in tubes of different shape or with different total volumes.

3d. Preparative fractionation of DNA in $\text{Hg}^{++}\text{-Cs}_2\text{SO}_4$

This was carried out in a manner similar to $\text{Ag}^+\text{-Cs}_2\text{SO}_4$ fractionation. The final concentration of DNA in 0.1 M Na_2SO_4 was usually 50 ug/ml. A 10^{-3} M solution of HgCl_2 was added to give Hg^{++} to

DNA-P molar ratios of 0.05 - 0.20. Saturated Cs_2SO_4 was added to give the required initial buoyant density and was adjusted accordingly.

Centrifugation, pooling of fractions and dialysis were carried out as described for $\text{Ag}^+ - \text{Cs}_2\text{SO}_4$ centrifugation.

4. Analytical centrifugation and determination of initial buoyant densities

Analytical centrifugation was carried out in the Beckman Model E Analytical Ultracentrifuge for 18 hours at 44 K rpm at 25°C. Ultra-violet photographs were taken and traced on the Joyce Loebel micro-densitometer.

The densities of the DNAs in neutral CsCl were determined from the position of a marker DNA (M. lys. DNA 1.731 gm/ml) added to the gradients.

The densities of the DNAs in alkaline CsCl were determined from the initial buoyant density of the solution and from the limiting isoconcentration distance (Vinograd and Hearst, 1962) according to the equation:

$$\rho_o = \rho + \alpha \omega^2 r (r_o - r_e)$$

where ρ_o = density of DNA at band centre

ρ = density of marker DNA or initial density of solution at isoconcentration point

α = (determined by the density gradient)

8.4×10^{-11} at 44 K rpm for CsCl at initial buoyant densities between 1.65 and 1.75 gm/ml (Chervenka, 1969)
or 1.4×10^{-11} at 44 K rpm for Cs_2SO_4 at initial buoyant densities of 1.35 - 1.40 gm/ml (Chervenka, 1969)

ω = angular velocity ($\omega^2 = 2.13 \times 10^7$ at 44 K rpm)

r_o = rotor centre to band centre

$r_e' = \sqrt{\frac{r_b^2 + r_a^2}{2}}$ where r_b = distance from rotor centre to the bottom of the cell

and r_a = distance from rotor centre to the meniscus in the cell

$$\bar{r} = \frac{(r_o + r_e')}{2}$$

5. Thermal Denaturation

Thermal denaturation and renaturation were carried out according to Speirs (1972) in a Unicam SP800 Spectrophotometer. Variable and constant temperatures were achieved with the Unicam temperature programmer (SP876) and heating block (SP877) attachments. Readings of optical absorbance were taken on a linear recorder (SP20) via a scale expander (SP 850). As the samples of DNA were usually small, Unicam microcells (10mm path length and 0.45 ml volume) were used to hold the samples. Cell holders were made to allow accurate alignment of the cells in the heating block. These cell holders were fitted with a clamping device to seal the cells and prevent evaporation.

The sample of DNA to be denatured and the reference sample of 0.1 x SSC were degassed by taking the samples up in a 1 ml syringe, blocking the needle with a rubber stopper, creating a vacuum in the syringe and tapping out the air bubbles. The cells were filled, fitted in the heating block and the temperature raised at 1°C/min until the hyperchromicity reached a plateau. The time was recorded manually every 2 minutes on the printout chart. Graphs were plotted as a percentage increase in hyperchromicity.

6. Thermal Renaturation

Renaturation was monitored in the same equipment used for denaturation. DNA samples were sonicated prior to melting and renaturation. DNA at 10 ug/ml in 0.1 x SSC was pulsed at full power (Dawe soniprobe) for 6 x 10 second pulses. The samples were kept on ice during the sonication and were allowed to cool for 1 minute between each pulse. The samples were then exhaustively dialyzed in 2 x SSC/50% formamide. A reference for each 2 x SSC/50% formamide system was provided by dialyzing an E.coli DNA standard together with the DNA samples. By obtaining the T_m of an aliquot of this standard before or after each melt, variations between experiments could be corrected. After dialysis the samples and the dialysate (which was used in the reference cell) were degassed to avoid the occurrence of bubbles on heating. Most optical measurements on nucleic acids are made at 260 nm. However, since formamide absorbs strongly at this wavelength, all denaturation and renaturation studies undertaken in this solvent were monitored at 270 nm. At this wavelength DNA still has 80% of its absorption at 260 nm while the formamide absorption is low.

The samples were sealed in narrow bore glass vials, heated to 90°C for 5 minutes to ensure full denaturation and rapidly cooled on ice, during which time the heating block and cells were equilibrated at the required temperature. The samples were quickly transferred to the preheated cells and zero time for renaturation was taken as the time the sample entered the cell. The cell was sealed, the spectrophotometer base line set to zero and the first reading then taken. Regular readings were taken throughout the renaturation.

Once sufficient renaturation had occurred, the sample was again melted, without removing it from the machine or altering any of the settings. This remelt supplied the true optical absorbance of the 100% denatured sample (A_{270} at 0 minutes), and the hyperchromicity of the sample was used to determine the 100% renaturation level. The data were plotted according to Britten and Kohne (1968).

7. RNA Transcription

Before being siliconised, all glassware was rendered RNase-free by washing in chromic acid and autoclaving at 110°C overnight. Radioactive nucleotides were purchased from The Radiochemical Centre, Amersham, and for the experiments reported, the nucleotides had the following activities: ATP, 20 Ci/mmole; CTP, 20.8 Ci/mmole; UTP, 14 Ci/mmole; GTP, 10 Ci/mmole. RNA polymerase was a gift from Dr J.O. Bishop. The optimum time of incubation for the polymerase reaction was determined by allowing the reaction to proceed for 1 hour with sampling at 10 minute intervals. The reaction plateaued after approximately 20 minutes. Since there should be no increase in TCA precipitable counts in a non-self-priming system the preparation of RNA polymerase was tested for self-priming by omitting template from a reaction mixture.

1. 0.1 mmoles of each of the four nucleotide triphosphates were lyophilized in a conical pyrex centrifuge tube (10 ml volume).

2. Approximately 20 ug of template DNA in 0.001 M NaCl was added and lyophilized.

3. The following solutions were stored frozen and added in the quantities indicated.

| Solution | Volume in ml | Molarity in final volume of 0.1 ml |
|----------------------------------|--------------|---------------------------------------|
| 1.0 M Tris pH7.5 at 30°C | 0.010 | 0.1000 |
| 0.005 M MnCl_2 | 0.010 | 0.0005 |
| 0.040 M MgCl_2 | 0.010 | 0.0040 |
| 0.020 M Spermidine | 0.008 | 0.0016 |
| 1.00 M KCl | 0.007 | 0.0700 |
| 0.020 M K_2HPO_4 | 0.010 | 0.0020 |
| distilled water | <u>0.043</u> | |
| | 0.098 | |

4. The mixture was thoroughly stirred on a Vortex mixer and a 1 μl sample spotted on an Oxoid membrane filter (2 cm grade 0.45). The sample provided a measure of the incorporation of radioactive nucleotides at zero time. 2 units of RNA polymerase (0.002 ml) was then added and mixed.

5. The transcription mixture was incubated in a 37° waterbath for 20 minutes, when another 1 μl sample was taken. The transcription mixture was chilled on ice.

6. The two Oxoid filters containing samples removed at 0 and 20 min time of incubation were washed 3 x for 5 min in ice-cold TCA, once for 5 min in absolute ethanol, oven-dried, and counted in toluene based scintillation fluid. An increase in TCA precipitable counts showed that the reaction mixture was working.

7. DNase was added to the transcription to a final concentration of 40 $\mu\text{g/ml}$ (4 μl of a 1 mg/ml solution made up in 0.01 Tris pH7.5, .002 M MgCl_2 and 10% dimethylsulphoxide and stored at -20°C) and the mixture incubated for 10 minutes at 37°C.

8. 10 μ l of 1 M NaCl (final concentration was 0.1 M NaCl) and 10 μ l of 5% SLS (final concentration was 0.1% SLS) were added and the mixture incubated at 37°C for 2 minutes.

9. Approximately 150 μ g of carrier RNA was added (5 μ l of a stock preparation of E.coli RNA in 0.1 x SSC).

10. An equal volume of distilled phenol in 0.1 x SSC was added and the mixture shaken vigorously for 5-10 minutes. After centrifugation the aqueous layer containing RNA was removed and the phenol washed twice with an equal volume of 0.1 x SSC. The three aqueous layers were pooled and placed directly on a G-50 Sephadex column.

11. The chromatography column (30 cm x 1.5 cm) was obtained from Pharmacia Fine Chemicals, Uppsala. Sephadex G-50 (Pharmacia, particle size 20 - 80 μ , bed volume of 10 ml/gm dry gel) was heated in an excess of 0.1 x SSC at 100°C for 1 hour and cooled. The excess 0.1 x SSC was decanted and the Sephadex solution poured slowly into the column, taking care that the pouring surface did not run dry. The column was equilibrated with 0.1 x SSC at 4°C overnight to allow firm packing of the column.

12. The RNA sample was pipetted onto the Sephadex interface and fractions eluted in 0.1 x SSC at a rate of 1 ml/5 min. 1 ml fractions were collected on an LKB fraction collector controller, type 3403 B.

13. The fractions were read spectrophotometrically at 260 nm. The first peak (eluting approximately at fraction 12 - 20) corresponds to RNA and the second peak (at 40 - 65) to the radioactive nucleotides.

14. TCA precipitable material was monitored by scintillation counting (as in step 6) in the fractions corresponding to the RNA peak.

15. The principal fractions containing RNA were pooled, lyophilized and taken up in sufficient distilled water to make the final solution 1 x SSC.

16. A 1 μ l sample of the final RNA product was TCA precipitated and counted (as in step 6). The efficiency of the counting machine was estimated using a 1 μ l sample of ATP of known specific activity. The specific activity of the RNA was estimated by assuming that all 4 nucleotides were incorporated in equimolar amounts in the RNA product.

8. Filter Hybridisation

Filter hybridisation was carried out essentially according to the method of Birnstiel et al (1968).

1. Gradients of DNA were prepared in the following way:

a. High molecular weight DNA was dissolved in 0.1 x SSC at a concentration of approximately 50 μ g/4 ml. 25 μ g of M. lys. DNA was added and CsCl (1.26 gm/ml) was added to form an initial buoyant density of 1.700 - 1.720 gm/ml. Tubes containing 5 ml of this solution were centrifuged to equilibrium in a 10 x 10 MSE angle rotor at 42 K rpm for 12 hours at 25°C. The tubes were punctured, ten drop fractions were collected, diluted with 0.5 ml of 0.1 x SSC and their absorbance read at 260 nm.

b. DNA dissolved in 0.1 M Na_2SO_4 was centrifuged in $\text{Ag}^+ - \text{Cs}_2\text{SO}_4$ essentially as described under "Preparative Fractionation of DNA in $\text{Ag}^+ - \text{Cs}_2\text{SO}_4$ ", except that the total amount of DNA per 5 ml tube was restricted to 70 μ g. Ten drop fractions were collected, diluted with 0.5 ml 10 mM EDTA, pH8.0 and their absorbance read at 260 nm. 0.05 ml of a 1 M KCN solution was added to each fraction to remove Ag^+ .

2. The fractions were then denatured by the addition of 0.5 ml of 1 M NaOH for 15 minutes at room temperature.

3. They were then neutralised with 2.5 ml of neutralising mix (1.0 M HCl, 1.0 M Tris buffer, pH8.0, 3.0 M NaCl, 1:1:2 by volume) and allowed to drip through 13 mm Millipore filters (HAWP 0.45 μ m pore size) which had been prewashed in 2 x SSC.

4. The loaded filters and blanks were washed with 6 x SSC, using approximately 5 ml/filter, dried in a vacuum oven at 80°C for 2 hours and stored at -20°C until use.

5. Hybridisation was performed with cRNA. The loaded filters were washed with 2 x SSC and then incubated at 60°C overnight in 0.7 ml of 2 x SSC containing between 3×10^5 and 1×10^6 cpm radioactive cRNA.

6. The filters were then chilled, washed in 1 change of 6 x SSC (2 liters) and 3 changes of 2 x SSC (2 liters each time), then RNased (10 μ g/ml in 1 liter of 2 x SSC) for 20 minutes at room temperature, washed in 2 changes of 2 x SSC (2 liters), dried in the vacuum oven at 80°C, and counted in 5 ml of toluene based scintillation fluid in a Nuclear Chicago Scintillation Counter. Machine counts were deducted.

9. In situ hybridisation

Three useful reviews of the subject are by Jones (1973), Hennig (in press) and Steffensen and Wimber (1972).

1. Chromosome spreads were prepared as described.

2. Coverslips (Chance, number 1, 22mm x 22mm) were cut to 11 x 11 mm size, washed in alcohol:ether, 1:1, siliconized (Repelcote, Hopkins and Williams, Chadwell Heath), dried, rinsed in distilled water and dried, ready for use.

3. A rubber sealing solution was prepared by mixing Cow Gum rubber solution (P.B. Cow, Ltd., Slough, Bucks) with sufficient benzene to form a barely pipettable solution.

4. The chromosome preparations were denatured, either in 0.2 M HCl (pH0.8) for 20 minutes or in 0.07 M NaOH (pH12.5) for 3-5 minutes. Denaturation in acid was favoured as it left the chromosomes in good cytological condition and easily stainable, although silver grains took longer to appear in the photographic emulsion. NaOH denaturation often resulted in "puffy" chromosome preparations which were more difficult to stain, but hybridisation results were achieved faster. Heat denaturation was generally avoided as the subsequent chromosome preparations often failed to stain, and the hybridisation reaction appeared to be less efficient even than that after HCl denaturation.

5. The chromosome preparations were dehydrated through 50%, 70%, 90% and absolute ethanol, then fan-dried at room temperature, ready for use.

6. Disposable rubber gloves were worn in this and the succeeding steps both to protect the skin from radioactivity and to protect the preparations from skin RNase. A large rubber bulb on a Pasteur pipette was used to blow dust from the chromosome preparations. Any trapped particulate material led to the trapping of air and subsequent patchy hybridisation.

7. Radioactive cRNA in 4 x SSC (2 - 6 x SSC) was applied to the denatured chromosome preparation with a Hamilton microliter syringe. Approximately 2 μ l was used if 1/4 size coverslips were used; approximately 5 μ l if full size coverslips were used.

8. Coverslips were carefully placed on the radioactive RNA and any trapped air gently tapped out. Rubber solution was pipetted around the edges of the glass coverslip to seal it to the glass slide.

9. The preparations were placed flat on a stainless steel tray which was floated in a 65°C waterbath, covered and incubated for 2-8 hours. The choice of incubating temperature depended on corresponding filter hybridisation experiments. In situ hybridisation experiments have been successful in 2 - 6 x SSC from 60 - 65°C with reaction times from 30 minutes to 16 hours. In the absence of experimental evidence for optimal incubation time an intermediate time was selected.

10. The rubber solution was removed and the coverslips lifted with a scalpel and forceps. The slides were placed in stainless steel racks and immediately immersed in 2 x SSC to prevent dehydration of the radioactive RNA on the chromosome preparation (leading to non-specific background labelling, or to less specific hybridisation as the salt concentration was increased through evaporation).

11. The slides were washed 3 x in 2 x SSC, then RNase (Sigma) (20 µg/ml in 2 x SSC, preheated to remove DNase and cooled) at 37°C for 30 minutes to remove non base-paired RNA.

12. The slides were rinsed in 2 x SSC, then washed in a stirred waterbath at 4°C in a vast excess of 2 x SSC for 2 - 4 hours.

13. The slides were dehydrated through 50%, 70%, 90% and absolute ethanol, then fan-dried at room temperature.

14. Autoradiography was carried out as described.

15. The slides were stored at 4°C in the dark for varying lengths of time (from 1 week to 6 months) before developing as described in the method for autoradiography.

16. Staining was carried out as described.

10. Autoradiography

1. Ilford K2 or Ilford L4 Nuclear Emulsion in gel form was used to coat slides for autoradiography. L4 has finer grains and thus the higher resolving power.

2. A specially constructed perspex container of approximately 25 ml volume and suitable for immersing a standard size slide (3 in x 1 in) was filled with 10 ml distilled water. Ilford emulsion which is supplied in shreds, was added to a measured 20 ml volume and the container placed in a $40 - 45^{\circ}\text{C}$ waterbath. The mixture was stirred to prevent local overheating and to thoroughly mix the melted emulsion and water. Care was taken to avoid causing a froth on the surface which would later prevent an even coating of the slides.

3. Slides were dipped in the melted emulsion and hung vertically to dry in a stream of air at 18°C .

4. They were then stored in the dark in light proof boxes at 4°C for varying lengths of time.

5. Slides were developed in Kodak D 19 B developer for $4\frac{1}{2}$ minutes at 18°C , washed in distilled water, fixed in Johnson Fix-Sol (diluted 1:5 with distilled water) for 5-10 minutes and stained.

11. Staining Slides

Staining was carried out either in toluidine blue or Giemsa (both from Gurrs, London).

Toluidine blue was used in a 0.02% solution made up in 0.001 M phosphate buffer (pH6.0). Slides were stained from 5 - 30 minutes, washed in buffer and air dried.

Giemsa R66 was used in one of two buffering solutions, pH5.75 or pH6.8. The former gives a better stain colour, the latter indicates chromosome banding patterns. Buffer pH5.75 was made by adding 85 ml of 0.1 M citric acid to 115 ml of 0.2 M Na_2HPO_4 and making up to 1 liter with distilled water. Buffer pH6.8 was made with Gurrs pH6.8 buffer tablets. Staining was carried out in a solution of 100 ml buffer, 5 ml Giemsa, and 3 ml methanol for 10 - 30 minutes. The slides were washed in buffer and air dried. Over-stained slides were destained in buffer.

Microscopy was carried out on a Zeiss Universal large research microscope and photographs were taken with a Hasselblad 500 C/M camera and Kodak panatomic X film.

12. Preparation of HAP

Hydroxyapatite was prepared essentially according to the method of Miyazawa and Thomas (1965).

1. 200 ml distilled water was poured in a 4 liter beaker.

2. 1 liter each of 0.50 M Na_2HPO_4 and 0.50 M CaCl_2 were added at a rate of 4.8 ml/min from 2 separatory funnels, whilst the contents of the beaker were mixed with a magnetic stirrer.

3. The precipitate formed was washed four times with distilled water, and was then suspended in 2 liters distilled water containing 50 ml 40% NaOH. This mixture was then boiled with simultaneous stirring for 1 hour.

4. The precipitate was washed a further four times with 2 liters distilled water.

5. Phosphate buffer was made with equimolar amounts of Na_2HPO_4 and NaH_2PO_4 , 1 M pH6.8.

6. The precipitate was washed in 2 liters 0.01 M PB and brought to boiling point with simultaneous stirring.

7. The precipitate was boiled in a further 2 liters of 0.01 M PB for 5 minutes with continual stirring.

8. The precipitate was boiled in a further 2 liters of 0.01 PB for 15 minutes with continual stirring.

9. The HAP was stored in 0.001 M PB with a small amount of chloroform at 4°C .

13. Use of the HAP to separate rapidly renaturing DNA

Instead of using HAP columns, the more convenient batch method of Flamm et al (1969b) was used.

1. The HAP was sheared in the Sorvall omnimixer at top speed for 1 minute to give uniform small crystal size to the preparation and to increase its capacity. Saturation for the preparation was found to be 500 μg DNA/gm HAP crystals, wet weight. The capacity of large crystals of HAP is normally very variable, and the shearing process makes the capacity reproducible.

2. Aliquots of HAP (slightly in excess of 1 gm) were placed in Quick Fit pyrex test tubes (8 ml volume) and placed in a 70°C waterbath. At the same time 500 ml quantities of 0.12 M PB and 0.30 M PB were maintained at 70°C.

3. The HAP aliquots were washed 3 x with 5 ml 0.12 M PB and resuspended in 4 ml 0.12 M PB ready for use (if not 4 ml, then sufficient PB such that buffer plus DNA sample in PB would make a total of 5 ml). The number of HAP tubes prepared would depend on the number of samples required to produce either a "cot plot" or a required quantity of rapidly renaturing DNA.

4. The DNA was dissolved in 0.12 M PB and was sheared to a uniform size by sonication (Dawe Soniprobe) with 5 one second pulses at top speed (number 3) and 1 minute cooling periods between pulses. The sample was kept at 0°C during sonication. This treatment gave a size of 300,000 daltons (determined by sedimentation analysis in the Model E).

5. DNA in 0.12 M PB was heat denatured at 100°C for 10 minutes and plunged into ice until the temperature reached 70°C. It was then rapidly placed in the 70°C water bath. For a cot plot a 1 ml sample was quickly removed and added to a prepared tube of HAP. The initial concentration of DNA was chosen so that either selected cot values were achieved at specific time intervals, or that the HAP was virtually saturated by rapidly renaturing DNA.

6. The remainder of the DNA was renatured at 70°C and at selected time intervals samples were removed to the prepared HAP tubes. If a quantity of DNA of a particular cot value was desired, and not a cot plot, then all samples of DNA were removed at the same time, after the

desired cot value had been achieved, and added to the prepared HAP tubes.

7. The samples were mixed by pipetting with a Pasteur pipette until the solution was homogeneous and no HAP precipitate remained at the bottom of the tube.

8. They were then equilibrated at 70°C for 1 minute and centrifuged at 2200 rpm for 15 seconds at 70°C to precipitate the HAP.

9. The supernatant was decanted and the HAP washed twice by pipetting (as in step 7) with 5 ml of 0.12 PB, then three times with 5 ml of 0.30 PB. The denatured DNA was recovered from the 0.12 M PB and the renatured DNA from the 0.30 M PB.

10. For a cot plot, the percentage renaturation was plotted against the cot value.

RESULTS

SECTION A. SATELLITE DNA IN THE HUMAN GENOME

Introduction

Britten (1969) and Saunders et al (1972b) measured the frequency of repetition in the human genome by successive hydroxyapatite fractionation. Their combined data indicates that approximately 10-20% of the DNA is rapidly renaturing (10^4 copies and a Cot value of 1 or less), approximately 20 to 30% is reiterated to a lesser extent (10^2 to 10^4 copies and a Cot of about 1 to 50) and the remaining 50 to 60% of the DNA consists of slowly renaturing and unique sequence DNA (approximately 10 copies or less and a Cot greater than 50). Using thermal elution from hydroxyapatite Hsu et al (1972) and Saunders et al (1972b) divided human DNA into fractions ranging from low to high GC and separated a rapidly reassociating DNA from each. They found that most rapidly reassociating sequences in human DNA had higher GC content than the bulk of the DNA, an unexpected finding since the rapidly renaturing fractions isolated as satellite DNAs have been AT rich (Corneo et al, 1967, 1968a,b, 1970a, 1971, 1972).

In situ localisation of human repetitious DNA was carried out by Arrighi et al, 1970b; Arrighi et al, 1971; Hsu et al, 1972 and Saunders et al, 1972a. They found that highly repetitious DNA was present in the entire complement and was not restricted to heterochromatic regions. When RNAs complementary to rapidly renaturing fractions of varying AT/GC ratios were hybridised, the higher GC content fractions were found to have comparatively less affinity for the centromeric regions. They suggested that human heterochromatin was composed of a variety of DNA families.

Table 2. Conditions for isolation and the physical properties of human satellite DNA fraction.

| Name of Fraction | % of genome | Tissue extracted from | Buoyant density | | | | Bd of sepd strands | T _m | enriched in heterochromatin |
|------------------|-------------|--|-----------------|-----------|--------|--------|--|-------------------------------|-----------------------------|
| | | | Main Band | Satellite | | | | | |
| Sat I | 1% | bone marrow, lymphnode, leucocytes, HeLa | 1.700 | Native | Denat. | Renat. | 1.707 1.738 | 80° C SSC | Yes |
| | | | | 1.687 | 1.704 | 1.694 | | | |
| Light Sat. | 0.2% | HeLa cultured diploid placenta, spleen, liver, | 1.698 | 1.686 | - | - | 1.731 1.764 plus d.s at 1.750 | - | - |
| Sat II | 2% | placenta. | 1.700 | 1.693 | 1.704 | 1.696 | 1.740 1.750 | 85° C in SSC | Yes |
| Sat III | 1.5% | placenta | 1.700 | 1.696 | 1.715 | 1.703 | 1.740 1.754 | 84° C in SSC | Yes |
| Sat IV | 2% | placenta | 1.700 | 1.700 | 1.716 | 1.705 | 1.730 1.742 | - | - |
| Homog. main-band | 15% | placenta | 1.700 | 1.696 | 1.715 | 1.709 | - | 87° (from graph) in SSC | - |
| Heavy Sat.(a) | - | HeLa | 1.698 | 1.712 | 1.774 | - | - | - | - |
| Heavy Sat.(b) | - | leucocytes (chronic lymphocytic leukemia) | 1.697 | 1.703 | - | - | - | - | - |

Table 2. Conditions for isolation and the physical properties of human satellite DNA fractions. 51a.

| enriched in nucleolar | Renaturation data | Method of Extraction | Ref. |
|-----------------------------|---|--|---|
| - | - | (1) 25 ug/ml in CsCl (2) $\text{Hg}^{++}\text{Cs}_2\text{SO}_4$, 50 ug/ml, $R_F=0.1$ (3) $\text{Ag}^+\text{Cs}_2\text{SO}_4$, 70 ug/ml, $R_F=0.2$ (4) MAK elution, $\text{Ag}^+\text{Cs}_2\text{SO}_4$, 60 ug/ml $R_F=0.23$. | Corneo <u>et al</u> 1967 Corneo <u>et al</u> 1968a Corneo <u>et al</u> 1971 Corneo <u>et al</u> 1972 |
| Yes | - | (1) 15 ug/ml in CsCl (2) Nucleolar preparations | Schildkraut and Maio, 1969 |
| - | 10 ug/ml for 5 hr at 65°C in 2xSSC. Cot of 5.6×10^{-1} yields 73% renat. | (1) $\text{Ag}^+\text{Cs}_2\text{SO}_4$, 50 ug/ml, $R_F=0.27$ (2) $\text{Ag}^+\text{Cs}_2\text{SO}_4$, 70 ug/ml, $R_F=0.20$ (3) MAK elution, $\text{Ag}^+\text{Cs}_2\text{SO}_4$, 60 ug/ml $R_F=0.23$ | Corneo <u>et al</u> 1970a Corneo <u>et al</u> 1971 Corneo <u>et al</u> 1972 |
| - | 10 ug/ml at 60°C in 2x SSC. Cot 5.6×10^{-1} = 63% renat. | (1) $\text{Ag}^+\text{Cs}_2\text{SO}_4$, 70 ug/ml, $R_F=0.20$ (2) MAK elution, $\text{Ag}^+\text{Cs}_2\text{SO}_4$, 60 ug/ml $R_F=0.23$ | Corneo <u>et al</u> 1971 Corneo <u>et al</u> 1972 |
| - | 5 ug/ml for 2hr 60°C in 2xSSC. Cot 1.1×10^{-1} yields 63% renat. | (1) MAK elution, $\text{Ag}^+\text{Cs}_2\text{SO}_4$, 60 ug/ml $R_F=0.23$ | Corneo <u>et al</u> 1972 |
| - | 10 ug/ml for 5 hr at 65°C in 2xSSC. Cot 5.6×10^{-1} yields 58% renat. | (1) $\text{Hg}^{++}\text{Cs}_2\text{SO}_4$, 50 ug/ml, $R_F=0.1$ (2) $\text{Ag}^+\text{Cs}_2\text{SO}_4$, 70 ug/ml, $R_F=0.20$ | Corneo <u>et al</u> 1970 Corneo <u>et al</u> 1971 |
| Yes | - | (1) Nucleolar preparations from HeLa CsCl, 1 ug/ml | Schildkraut and Maio, 1969 |
| - | 72.9°C in 0.025 M Tris HCl pH8.0 | $\text{Ag}^+\text{Cs}_2\text{SO}_4$, $R_F=0.27$ | Saunders <u>et al</u> 1972a |

The human genome in neutral CsCl centrifugation does not separate into satellite and mainband fractions. However, a small light satellite is sometimes seen in the analytical ultracentrifuge when the centrifuge cell is heavily overloaded with DNA (Corneo et al, 1967; Schildkraut and Maio, 1969). With the more sophisticated technique of chelating heavy metal ions to DNA and banding it in Cs_2SO_4 density gradients Corneo isolated and characterized four human satellite fractions (see Table 2). In addition Schildkraut and Maio (1969) found a light¹ and a heavy satellite in HeLa DNA and Saunders et al (1972a) found a heavy satellite² in one leukemic patient (see Table 2).

RNA complementary to human satellite II hybridised predominantly to the centromeric regions of three pairs of chromosomes, 1, 9 and 16. Longer exposure intervals led to hybridisation in minor amounts to the centromeres of several other chromosome pairs, particularly the acrocentric chromosomes (Jones and Corneo, 1971). In situ hybridisation of human satellites III and I will be discussed in this report.

Saunders et al (1972a) located a satellite fraction with a buoyant density of 1.703 gm/ml in the centromeric heterochromatin of chromosome 9 and found some hybridisation to the centromeres of the D and G group chromosomes.

The following sections describe the isolation, characterization and hybridisation results of human satellites III and I, together with similar studies on the satellites in three other primate species.

-
1. similar to satellite I (Corneo et al, 1967)
 2. similar to satellite III (Corneo et al, 1970a) in its in situ localisation (Saunders et al, 1972a; Jones et al, 1973).

Figures 1 and 2. The isolation of human satellite III

Fig. 1 shows the optical density profiles of the preparative gradients used in the isolation of human satellite III.

Fig. 2 shows microdensitometer tracings of samples taken from the preparative gradients and analytically centrifuged in the Model E. The satellite was isolated according to the method described on page 53.

Fig. 1A. Human satellite III was centrifuged in $\text{Ag}^+ - \text{Cs}_2\text{SO}_4$,

$R_F = 0.20$, $70 \mu\text{gDNA/ml}$. III refers to the fractions of the gradient containing satellite III; I refers to the fractions containing satellite I.

Fig. 2a. A sample of the preparative gradient in 1A analytically centrifuged to show the positions of satellites III and I.

Fig. 1B. Fractions 20-30 from Fig. 1A were recentrifuged in CsCl .

III and I refer to the fractions containing these satellites determined from analytical centrifugation of samples across the gradient and illustrated in Fig. 2b.

Fig. 2b. B-22 and B-28 are microdensitometer tracings of samples from fractions 22 and 28 of the gradient illustrated in 1B.

M. lys. DNA, buoyant density = 1.731 gm/ml was added as a marker.

Fig. 1C. Fractions 24 to 28 from Fig. 1A were recentrifuged in CsCl .

III and I refer to the fractions containing these satellites determined from analytical centrifugation of samples across the gradient and illustrated in Figure 2c.

Fig. 2c. C-11, C-19 and C-24 are microdensitometer tracings of samples from fractions 11, 19 and 24 of the gradient illustrated in Figure 1C.

Fig. 1D. Fractions 19 to 23 from Fig. 1B and fractions 11 to 18 from Fig. 1C were recentrifuged in CsCl. Fractions containing pure satellite III were determined from analytical centrifugation of samples across the gradient and are illustrated in Figure 2f.

Fig. 2d. A sample of the preparative gradient in Fig. 1D was analytically centrifuged to show the overall composition of the DNA.

Fig. 1E. Fractions 24 to 29 of Fig. 1B and fractions 19 to 23 of Figure 1C were recentrifuged in CsCl. Fractions containing pure satellite III, and satellite I contaminated by satellite III, were determined from analytical centrifugation of samples across the gradient and are illustrated in Fig. 2g.

Fig. 2e. A sample of the preparative gradient in Fig. 1E analytically centrifuged to show the overall composition of the DNA.

Fig. 1F. Pure satellite III from the pooled fractions 31 to 37 of Fig. 1D and 29 to 33 of Fig. 1E. M. lys. DNA, buoyant density = 1.731 gm/ml, was added as a marker.

Fig. 1G. Satellite III and I from the pooled fractions 24 to 26 of Fig. 1C and 34 to 38 of Fig. 1E.

Fig. 2f. D-27, D-29 and D-31 are microdensitometer tracings of samples from fractions 27, 29 and 31 of the gradient illustrated in Fig. 1D.

Fig. 2g. E-29 and E-34 are microdensitometer tracings of samples from fractions 29 and 34 of the gradient illustrated in Fig. 1E.

Figure 1.

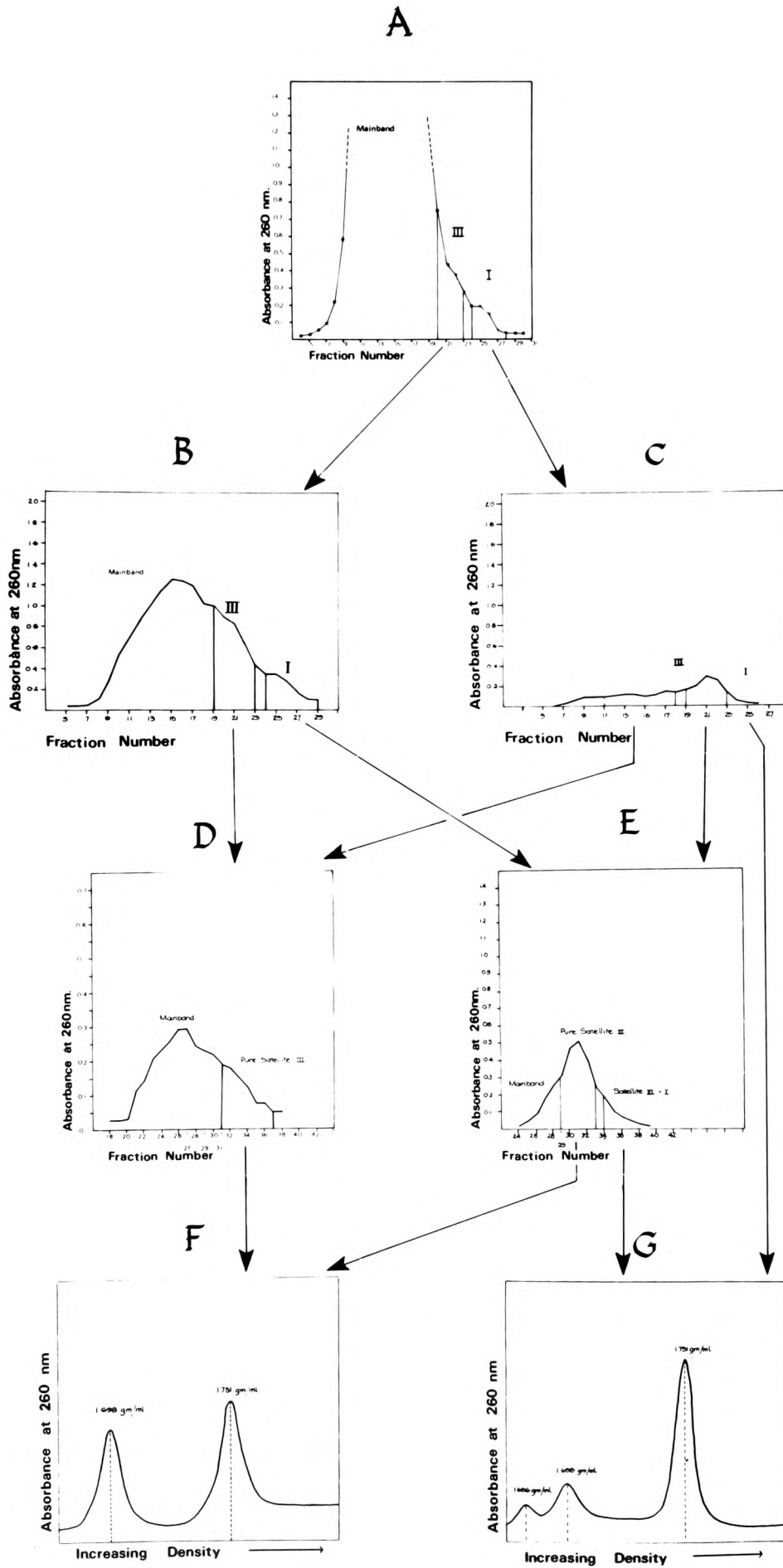
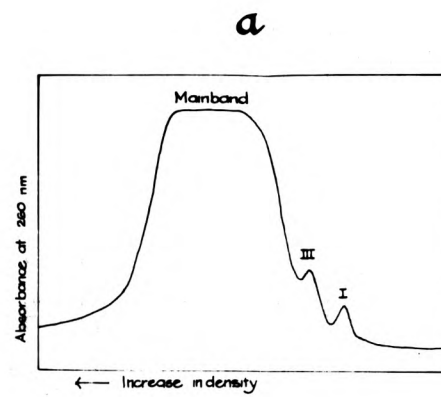
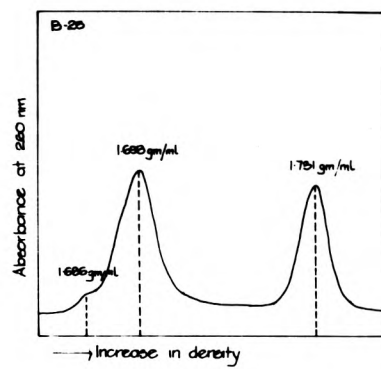
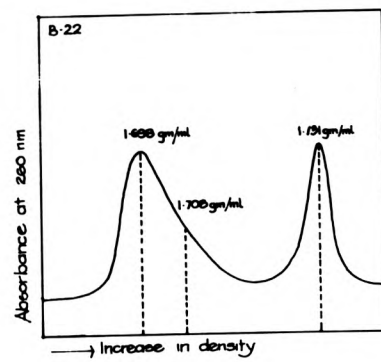


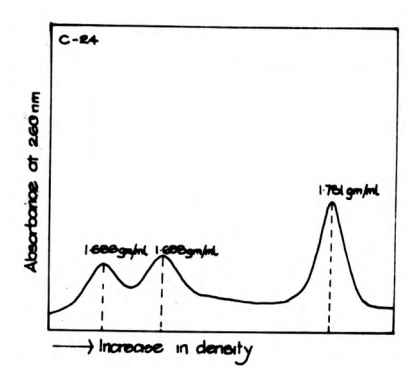
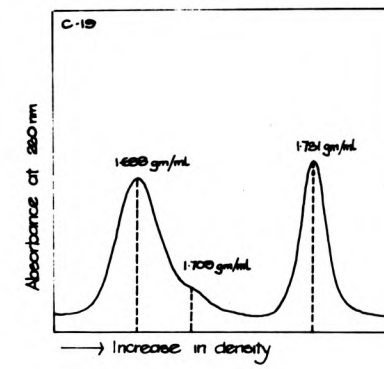
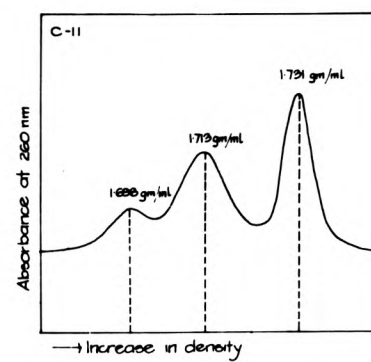
Figure 2.



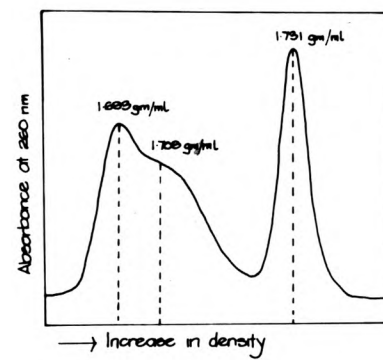
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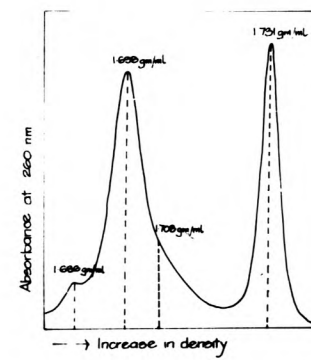
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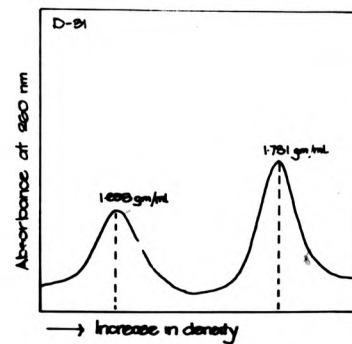
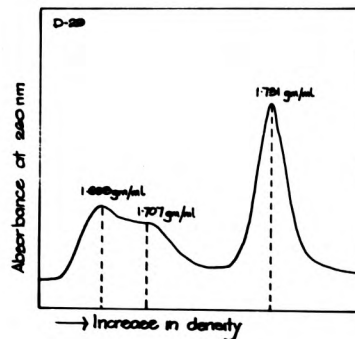
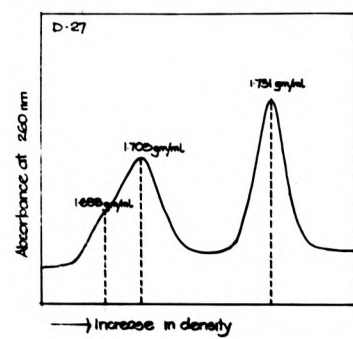
d



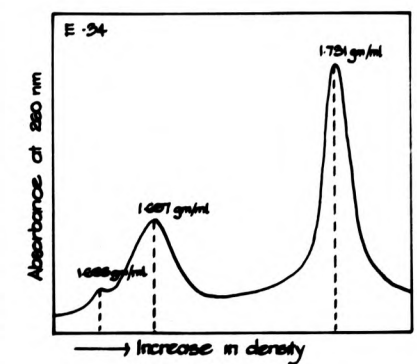
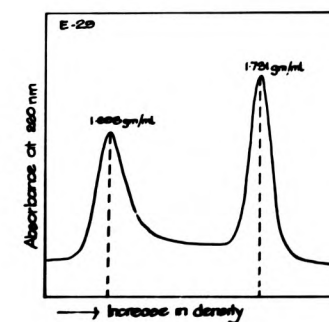
e



f



g



Satellite III

1. Preparation

The techniques of isopycnic centrifugation in CsCl and Cs_2SO_4 are outlined on pages 31 to 36. The following is an account of the results achieved at each step of the preparation. To isolate human satellite III, DNA was centrifuged in $\text{Ag}^+-\text{Cs}_2\text{SO}_4$ using 70 ug/DNA/ml and a molar ratio of 0.20. Eight tubes of 20 ml/tube were centrifuged in the 8 x 50 rotor of the MSE preparative ultracentrifuge at 20°C for 96 hours. 8-drop fractions were collected and their absorbance at 260 nm determined (without prior dilution) in the Beckman spectrophotometer using cells of 1 mm path length and .25ml capacity. Figure 1A shows the OD profile of one of the tubes. On the light side of the mainband were found two shoulders in which were located satellites III and I. Figure 2a shows a microdensitometer tracing of a sample from the same preparation which was analyzed in the analytical ultracentrifuge. The fractions from each of these two shoulders were pooled and recentrifuged in the 10 x 10 rotor for 40 hours at 44K. 6-drop and 4-drop fractions were collected, diluted and their absorbance determined in the Unicam spectrophotometer. Graphs 1B and 1C show the OD profiles of the two shoulders and from these, selected fractions (B-22, B-25, C-11, C-19, C-24) were analyzed in the analytical ultracentrifuge, the tracings of which are shown in Figures 2b and 2c. On the basis of the composition of these samples the fractions in 1B and 1C were pooled as indicated. The pooled fractions were dialyzed to remove the Ag^+ ions, concentrated and dialyzed into 0.1 x SSC.

The pooled fractions from Figures 1B and 1C were centrifuged preparatively in analytical grade CsCl for 40 hours at 44K in the 10 x 10 rotor for the final purification of human satellite III. Figures 2d and 2e show microdensitometer tracings of samples from each of the preparations which were analyzed in the analytical ultracentrifuge. 6-drop fractions were collected, diluted and their absorbance at 260 nm determined in the Unicam spectrophotometer. Figures 1D and 1E show the fractionation of these two gradients. Selected fractions (D-27, D-29, D-31, E-29, E-34) were analyzed in the Model E (Figures 2f and 2g) to determine the composition of the gradients. From these results the fractions indicated in Figures 1D and 1E were pooled to yield pure satellite III DNA and a fraction composed of Satellite III and I in approximately equal proportions (Figures 1F and 1G). The satellite fractions were concentrated by evaporation, dialyzed into 0.1 x SSC and stored at -20°C . 45 ug of pure human satellite III was prepared in this way.

2. Characteristics

The buoyant density (Figure 3) and thermal denaturation data (Figure 4) for human satellite III are as follows:

Buoyant density in neutral CsCl (gm/ml)

| | |
|---------------------------------|-----------------|
| Native | 1.698 |
| Denatured | 1.712 |
| Renatured to a Cot of 10^{-3} | 1.705 and 1.712 |

T_m in 0.1 x SSC

| | | |
|---|----------------------|----------------------|
| Native | 70°C | |
| Percent increase in hyperchromicity | 35% | |
| Renatured (uncorrected and corrected for single strand hyperchromicity) | Uncorrected | Corrected |
| | 63°C | 65°C |
| Percent increase in hyperchromicity | 33% | 24% |

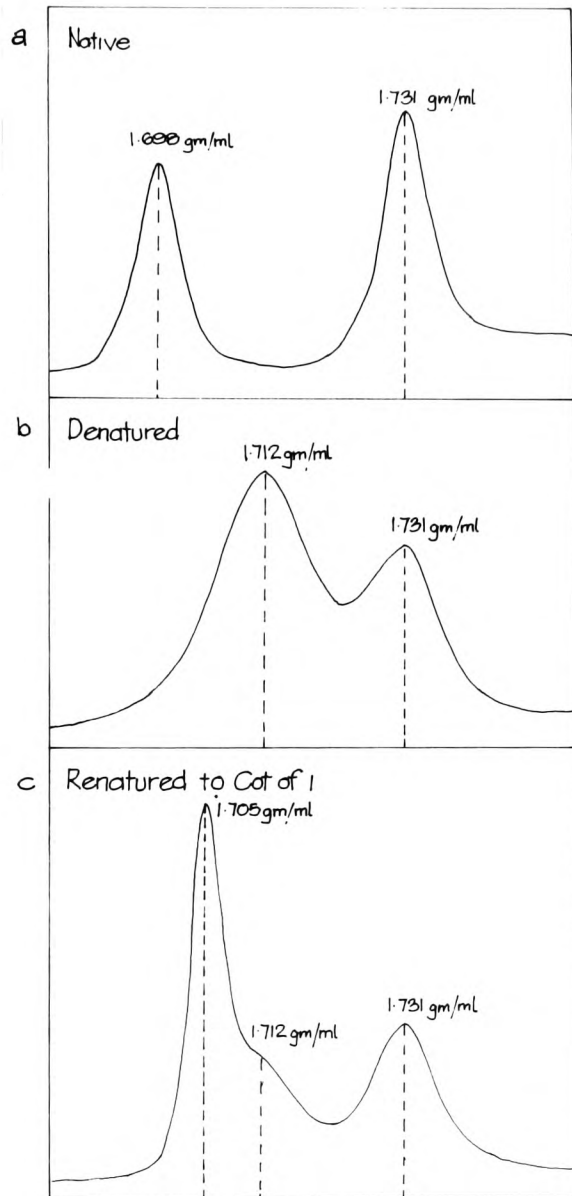


Fig. 3. Microdensitometer tracings of human satellite III DNA, centrifuged to equilibrium in the analytical ultracentrifuge (a) in neutral CsCl (b) denatured by heating at 100°C for 10 min. in 0.1 x SSC followed by rapid cooling (c) renatured at 2 µg/ml at 65°C for 3 min. in 2 x SSC after heat denaturation as in (b).

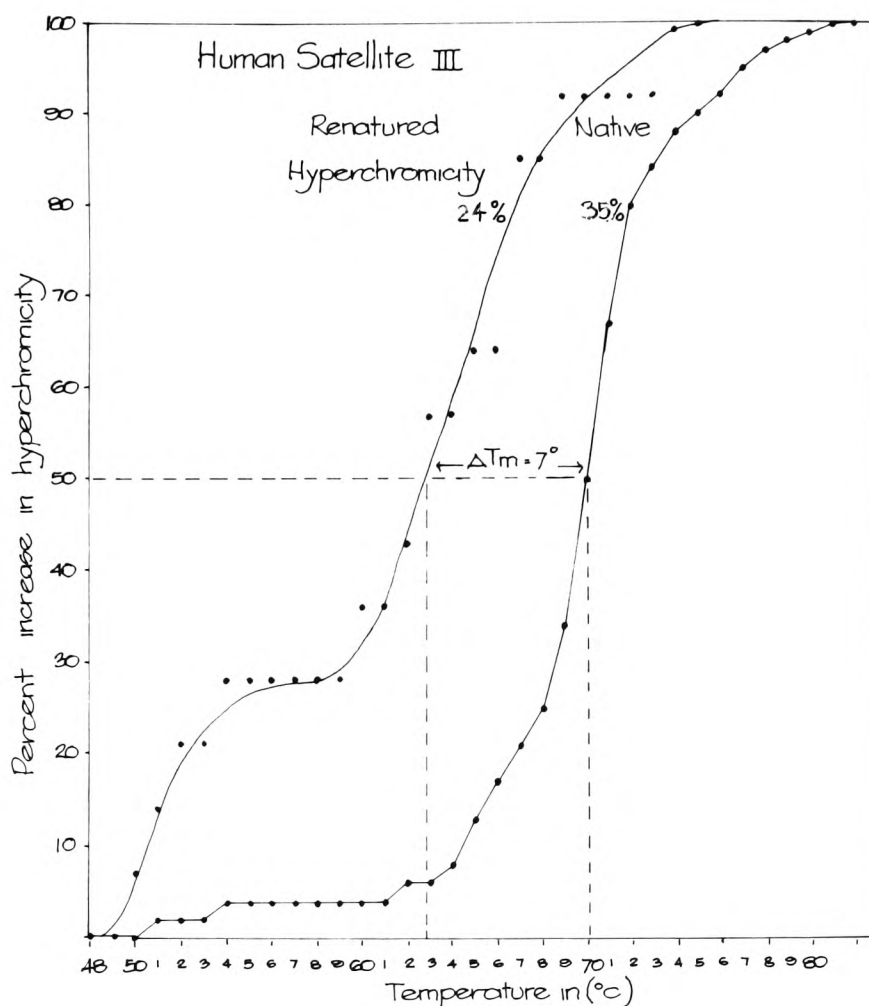


Fig. 4. The melting curves of native and renatured human satellite III in 0.1 x SSC. The renatured satellite sample was first denatured in 0.1 x SSC at 100°C for 10 min then made up to 2 x SSC and renatured for 3 min at 2 μ g/ml at 65°C. It was dialyzed into 0.1 x SSC and concentrated before melting. The conditions for thermal denaturation are described on page 37. The ΔT_m between native and renatured satellite DNA is 7° (uncorrected for single strand hyperchromicity) or 5° (corrected).

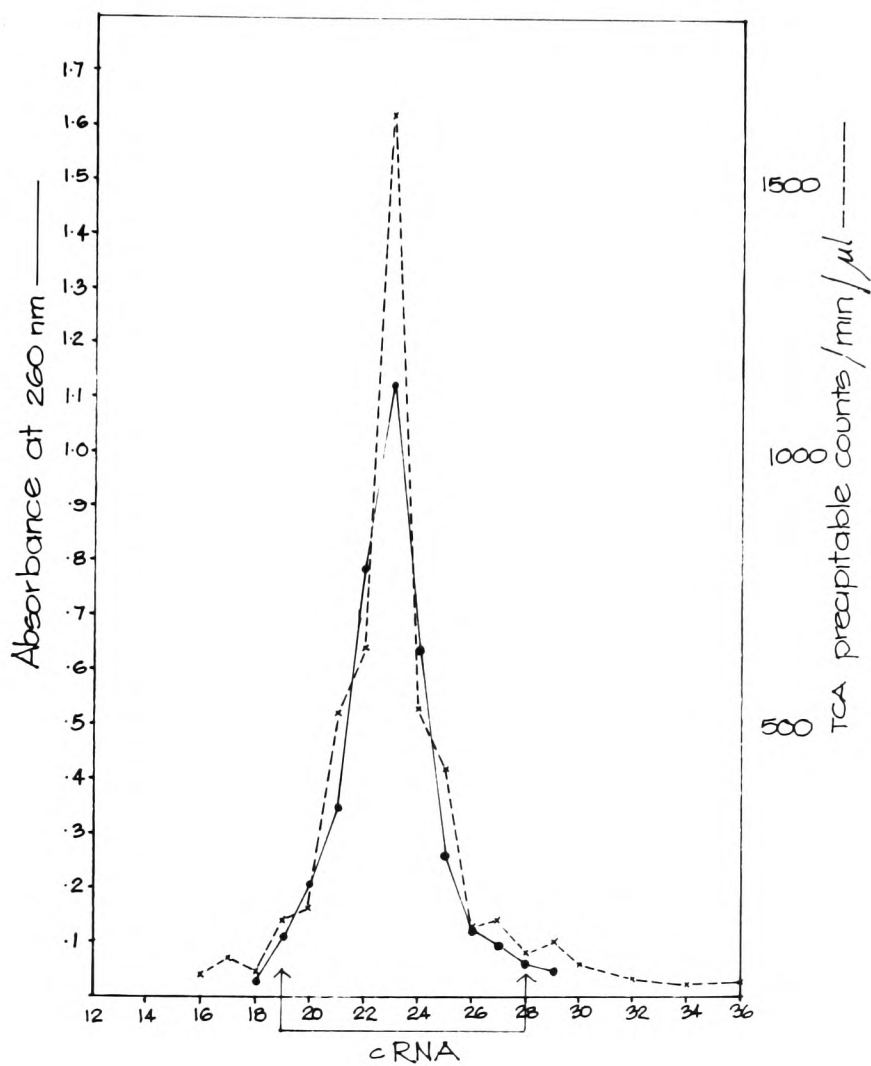


Fig. 5. The peak of radioactive satellite III crRNA eluted from a Sephadex G-50 column according to the method described on page 39.

Thermal denaturation profiles were obtained for 7.5 ug of native and Cot 1 renatured satellite melted in 0.1 x SSC (Figure 4). The renatured sample was prepared as described in the figure legend. Plotted as indicated in Figure 4 a ΔT_m of 7° was obtained. However, the microdensitometer tracing of Cot 1 satellite III analyzed in the Model E (Figure 3c) indicates that a proportion of the sample remains single stranded. It is probable that the initial phase of the Cot 1 melting curve, which constitutes 28% of the total increase in OD, is due to single strand hyperchromicity. If the second phase (72% of the total increase in OD) were due to melting of the renatured duplex, the increase in hyperchromicity for the Cot 1 sample is then 24% and the T_m is 65°C , giving a ΔT_m of 5° .

3. Transcription

Human satellite III was transcribed using 20 ug of template and all four nucleotides radioactive. Figure 5 shows the peak of cRNA eluted from the Sephadex G-50 column. Fractions 19-28 were pooled, lyophilised and redissolved in distilled water to make 0.25 ml of human satellite III cRNA in 4 x SSC with a specific activity of 1.1×10^8 dpm/ug. The cRNA contained 2.5×10^4 cpm/ul.

4. Gradient hybridisation

Satellite III cRNA was hybridised to human DNA which had been fractionated in Cs salt density gradients. The composition of the gradients and hybridisation details are indicated in the figure legends. Satellite III cRNA hybridised to human DNA from a CsCl gradient to a fraction with a buoyant density of $1.696 \text{ gm/ml} + .002 \text{ gm/ml}$ (Figure 6). This result was in accordance with expectations as the original template satellite had the same buoyant density. Hybridisation to human DNA

from an $\text{Ag}^+ - \text{Cs}_2\text{SO}_4$ gradient, $R_F = 0.20$ (Figure 7) was with a fraction of DNA in the region of satellite III (see Figure 2a for a microdensitometer tracing of the relevant gradient showing the positions of satellites III and I). Hybridisation also occurred with DNA located in the "mainband" peak. This may be because there is satellite III DNA which does not separate from the mainband in the $\text{Ag}^+ - \text{Cs}_2\text{SO}_4$ method employed, or it may mean that there are sequences in the mainband which are sufficiently like the sequences of satellite III to hybridise with the crRNA but sufficiently different from them to complex the Ag^+ ion to a greater extent. The CsCl gradient hybridisation does not distinguish between these possibilities since the hybridisation peak is wide and encompasses hybridisation to the mainband DNA.

It is also possible that the hybridisation of satellite III crRNA to DNA in the mainband is due to sticking of the crRNA to a high concentration of DNA on the filters, although it was not seen to occur to M. lys. DNA in Figure 6.

5. In situ hybridisation

Human satellite III crRNA was hybridised to human and mouse chromosomes by the technique described in methods. 1 μl /slide was used and hybridisation was carried out at 65°C for 12 hours. The slides were dipped in Ilford L4 liquid emulsion and stored at -4°C for two weeks to two months.

Human satellite III crRNA was found localised in human chromosomes in the pericentromeric heterochromatin of chromosomes pair 9 (Figure 8). The identification of the chromosome was confirmed by using preparations which contained a pericentromeric inversion of the heterochromatic

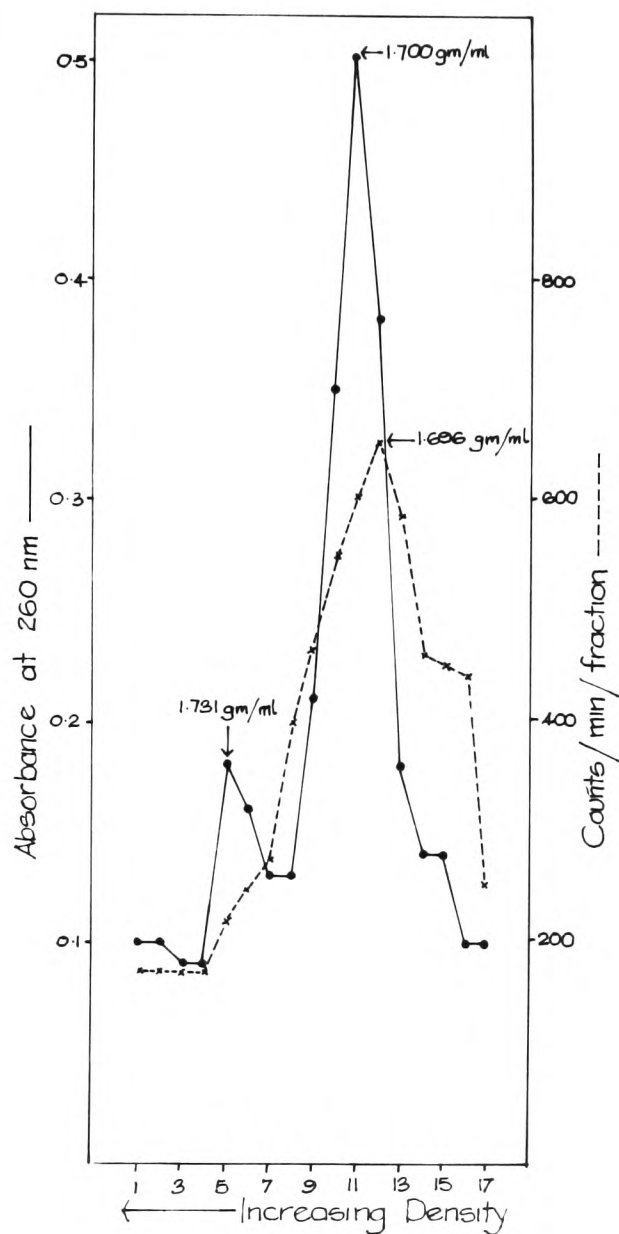


Fig. 6. Filter hybridisation result of human satellite III cRNA to human DNA fractionated in CsCl. The conditions for hybridisation are described on page 42. $1.1 - 1.2 \times 10^5$ counts/min of satellite III cRNA was added to 0.5 ml of incubation mix and hybridisation took place at 68°C for 4 hours. The hybridisation was carried out by M. Moar.

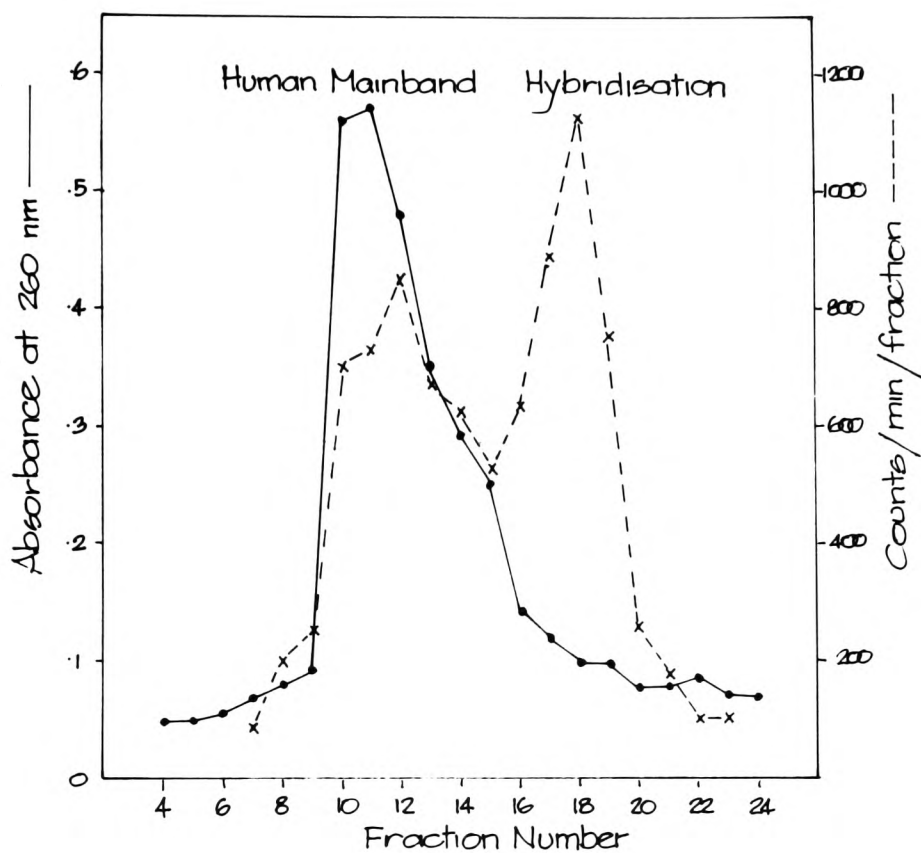


Fig. 7. Filter hybridisation result of human satellite III cRNA to human DNA fractionated in $\text{Ag}^+ - \text{Cs}_2\text{SO}_4$ $R_F = 0.20$. The conditions for hybridisation are described on page 42. 2.1×10^5 counts/min of satellite III cRNA was added to 0.7 ml of incubation mix and hybridisation took place in 65°C overnight.

(a)

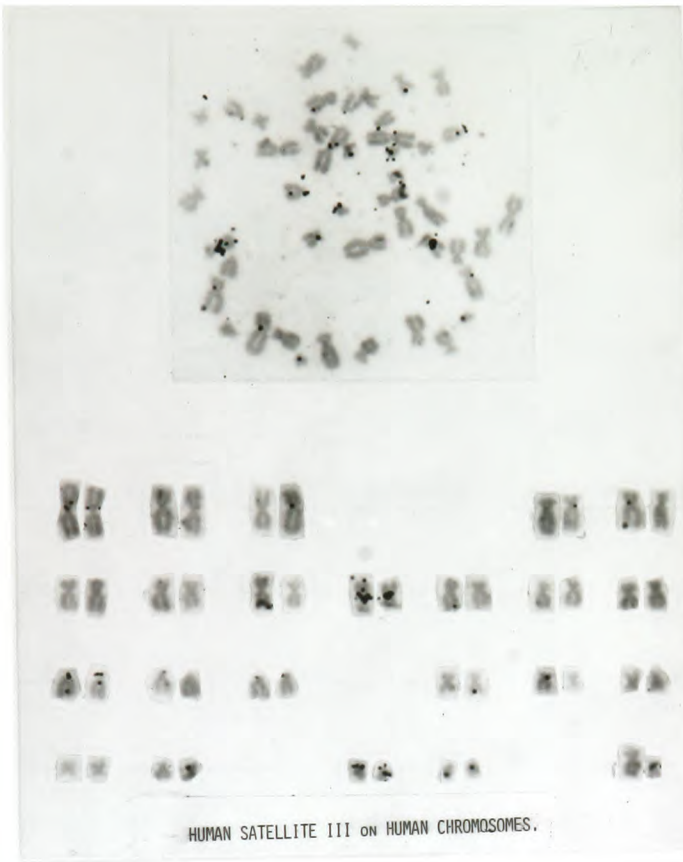


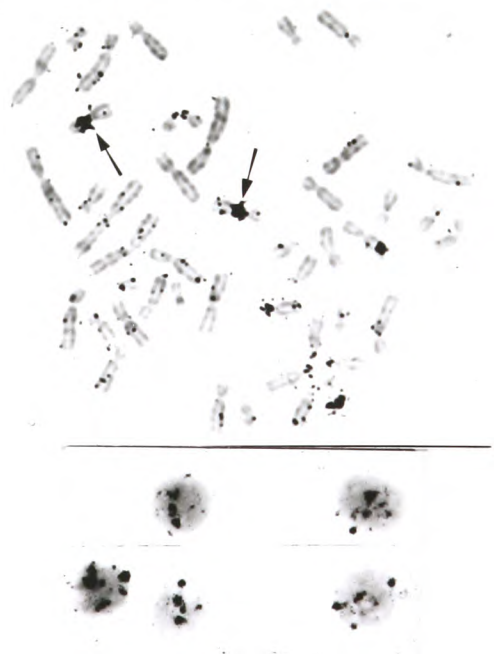
Fig. 8.

In situ hybridisation of human satellite III cRNA to human metaphase chromosomes, according to the method described on page 43.

(a) Normal karyotype

(b) Chromosomes from a subject carrying a pericentromeric inversion of the heterochromatin on chromosome 9.

(b)



region of the chromosome in question. The centromeres of several small chromosomes, particularly of the D and G groups, were involved in the hybridisation to a lesser extent. Interphase nuclei showed a concentration of grains over heterochromatic areas indicating that the areas of localisation are condensed and to some extent associated in interphase. Mouse chromosomes showed only background levels of randomly distributed grains.

Satellite I

1. Preparation

Human satellite I was prepared by centrifuging human DNA in a Hg^{++} - Cs_2SO_4 density gradient using 50 μg DNA/ml and a molar ratio of Hg^{++} :DNA-P of 0.10. Four tubes of 20 ml/tube were centrifuged in the 8 x 50 rotor of the MSE 50 ultracentrifuge. 8-drop fractions were collected, diluted and their absorbance at 260 nm determined in the Unicam spectrophotometer. Figure 9 shows the OD profile of the relevant side of the gradient together with a microdensitometer tracing of a sample from the preparation centrifuged in the Model E. The satellite clearly separates from the mainband on the heavy side of the gradient, and therefore fractions from the bottom of the tube were collected only until the mainband emerged. Fractions 12 to 16 and analogous fractions from the remaining tubes were pooled, dialyzed to remove Hg^{++} ions and stored in 0.1 x SSC at -20°C . 16 μg of pure human satellite I was prepared in this way (Figure 10). 16 μg constitutes 0.4% of the initial 4 mg of DNA preparatively centrifuged. Since satellite I separates distinctly from the mainband, this is probably a reasonable estimate of its proportion in the human genome.

2. Characteristics

The buoyant density (Figure 11) and thermal denaturation data (Figure 12) for human satellite I are as follows:

| Buoyant density in neutral CsCl (gm/ml) | |
|---|---------------------------------------|
| Native | 1.686 |
| Denatured | 1.699 |
| Renatured to a Cot of 10^{-3} | 1.689 |
| Separated single strands in alkaline CsCl | 1.736 and 1.766 plus peak at 1.758 |
| T_m in 0.1 x SSC | |
| Native | 60°C |
| Percent increase in hyperchromicity | 31% |
| Renatured | 55°C |
| Percent increase in hyperchromicity | 24% |

Thermal denaturation profiles were obtained for 7 ug of the native and Cot 1 renatured satellite DNA melted in 0.1 x SSC (Figure 12). Cot 1 renatured satellite I was prepared as described in the legend for Figure 4.

3. Transcription

Human satellite I was transcribed using 10 ug of template and all four nucleotides radioactive. Figure 13 shows the peak of cRNA eluted from a Sephadex G-50 column. Fractions 19-24 were pooled, lyophilised and redissolved in distilled water to make 0.135 ml of human satellite I cRNA in 4 x SSC with a specific activity of 1.06×10^7 dpm/ug. The cRNA contained 3.8×10^4 cpm/ul.

4. Gradient hybridisation

Satellite I cRNA was hybridised to human DNA which had been fractionated in a CsCl density gradient. The composition of the gradient and hybridisation details are indicated in the figure legend. Satellite I cRNA hybridised to a fraction of DNA with a buoyant density of $1.688 \text{ gm/ml} \pm 0.002 \text{ gm/ml}$ (Figure 14). This result was in accordance

with expectations as the original template satellite had a buoyant density of 1.686 gm/ml.

5. In situ hybridisation

Satellite I crRNA was hybridised to human and mouse chromosomes, denatured in NaOH and HCl as described in methods, and in 90% formamide/0.1 x SSC for $2\frac{1}{2}$ hours at 65°C (Steffensen and Wimber, 1972). 5 ul/slide of crRNA was used and hybridisation was carried out at 65°C for 4 hours. The slides were coated with Ilford L4 liquid emulsion and stored at -4°C for 5 weeks. Before developing the slides were dipped in a latent image intensification bath (Jacob, 1971) for 15 minutes and then developed as usual.

Human satellite I was found localised in the large block of heterochromatin on the distal arm of the Y chromosome and in the heterochromatin of a C group chromosome, probably chromosome 9 (Figure 15). Hybridisation was seen in the slides denatured in formamide but not in those pH denatured in the usual way. For reasons which are unclear, formamide may be a more efficient means of denaturation of some DNAs. There was no hybridisation in the heterologous reaction with mouse chromosome.

Rapidly Renaturing DNA

Human DNA renatured to a Cot of 10 was separated from denatured DNA by the HAP batch method as described. The Cot 10 DNA was used as a template for in vitro transcription of crRNA and produced a poor yield of low specific activity crRNA, perhaps due to the fact that the DNA was highly sheared. The in situ hybridisation result (Figure 16) shows hybridisation of RNA made complementary to rapidly renaturing human DNA randomly distributed on all the chromosomes with no specific localisation.

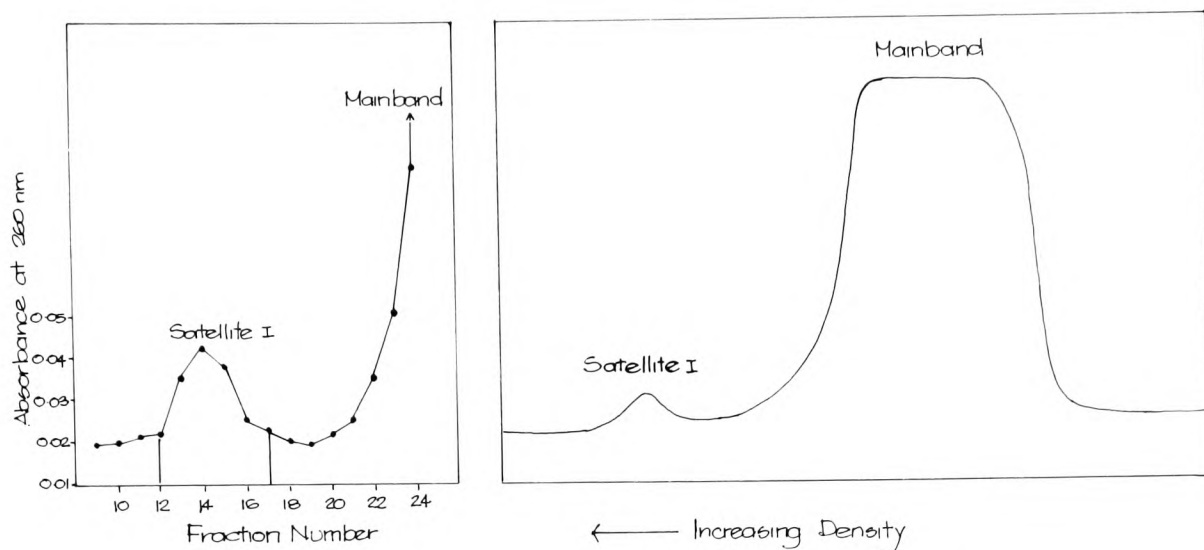


Fig. 9. Preparative (a) and analytical (b) equilibrium density gradient centrifugation of human total DNA in $\text{Hg}^{++}\text{-Cs}_2\text{SO}_4$, $R_F = 0.10$. The method is described on page 57.

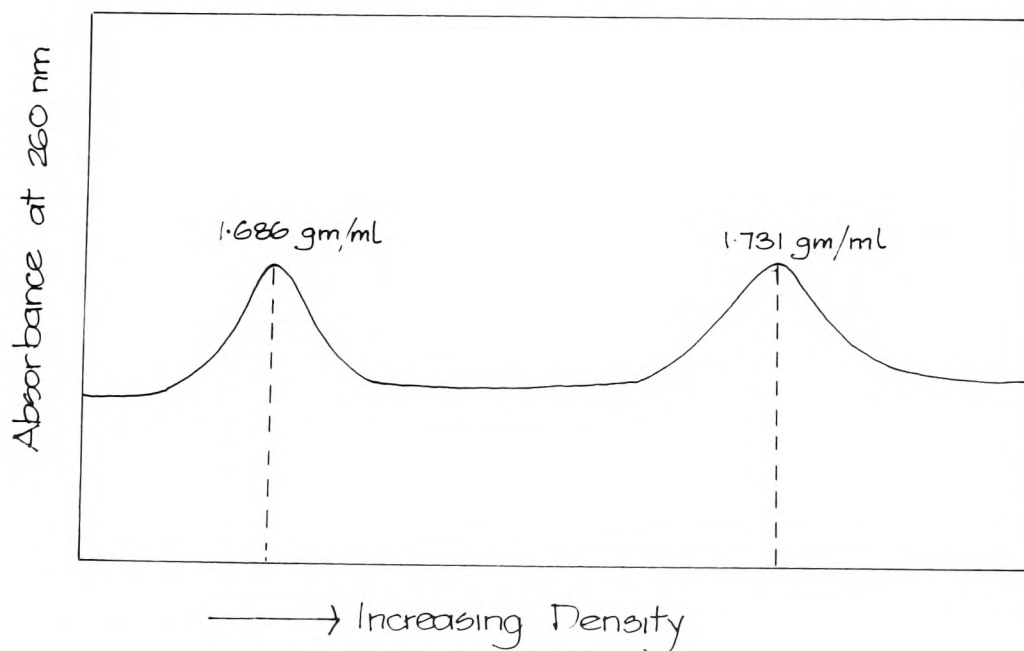


Fig. 10. Pure human satellite I centrifuged to equilibrium in neutral CsCl . M. lys. buoyant density = 1.731 gm/ml was added as a marker.

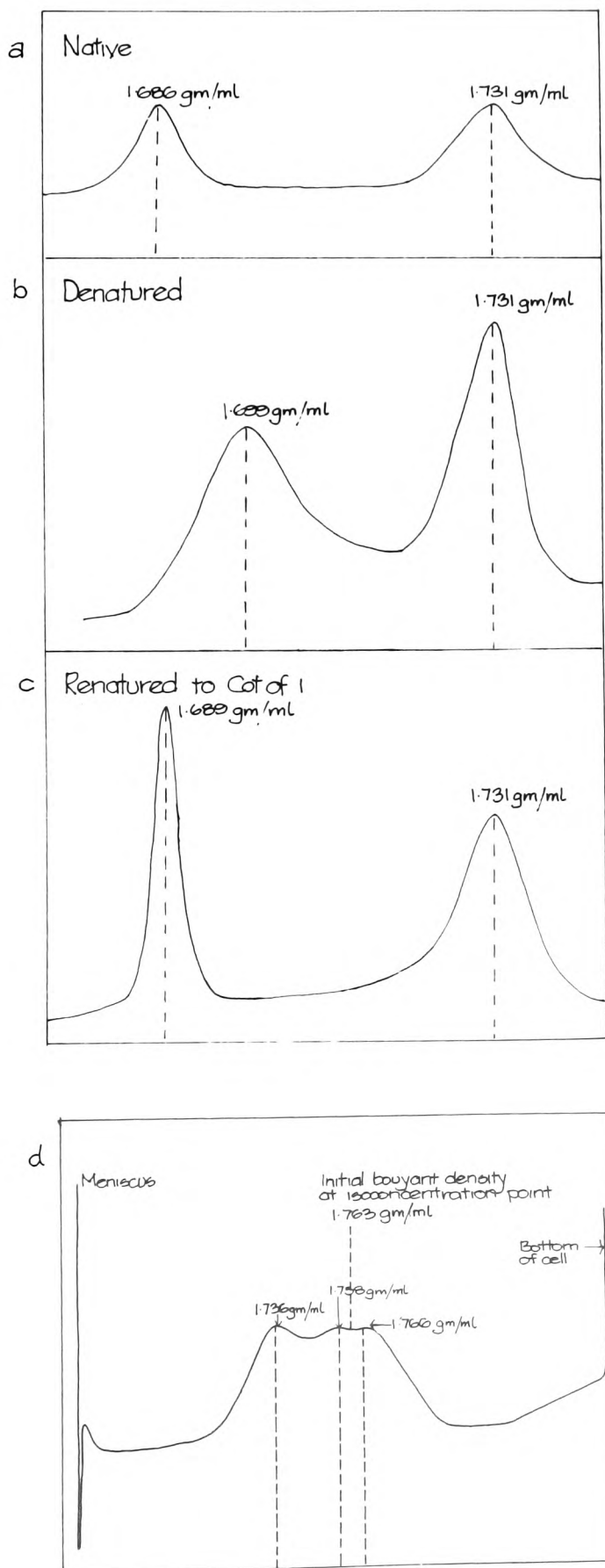


Fig. 11. Microdensitometer tracings of satellite I DNA centrifuged to equilibrium in the analytical ultracentrifuge in neutral CsCl (a) native (b) denatured by heating at 100°C for 10 min. in $0.1 \times \text{SSC}$ followed by rapid cooling (c) renatured at $2 \mu\text{g/ml}$ for 3 min. in $2 \times \text{SSC}$ after heat denaturation as in (b). M. lys. DNA, buoyant density = 1.731 gm/ml was added as a marker. (d) Satellite I DNA centrifuged to equilibrium in alkaline CsCl. Density in alkaline CsCl was determined from the limiting isoconcentration distance and the initial density of the solution.

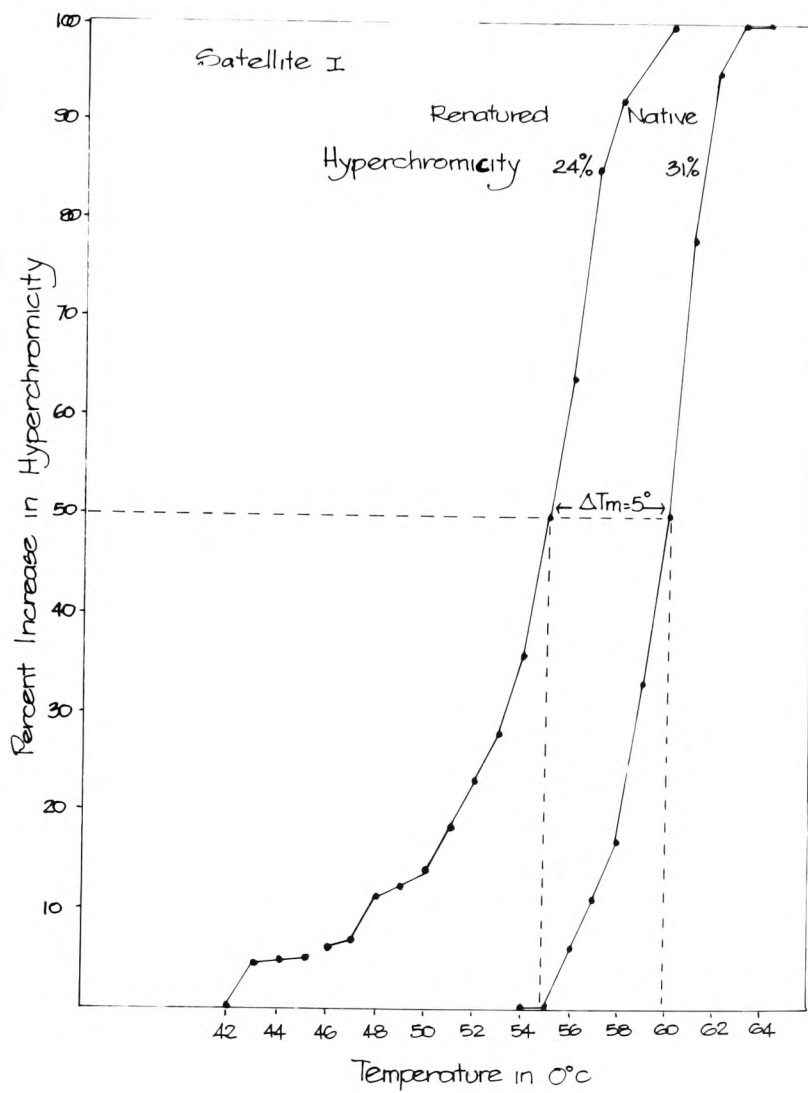


Fig. 12. The melting curves of native and renatured satellite DNA in 0.1 x SSC. The renatured satellite was prepared as described in the legend for Fig. 4.

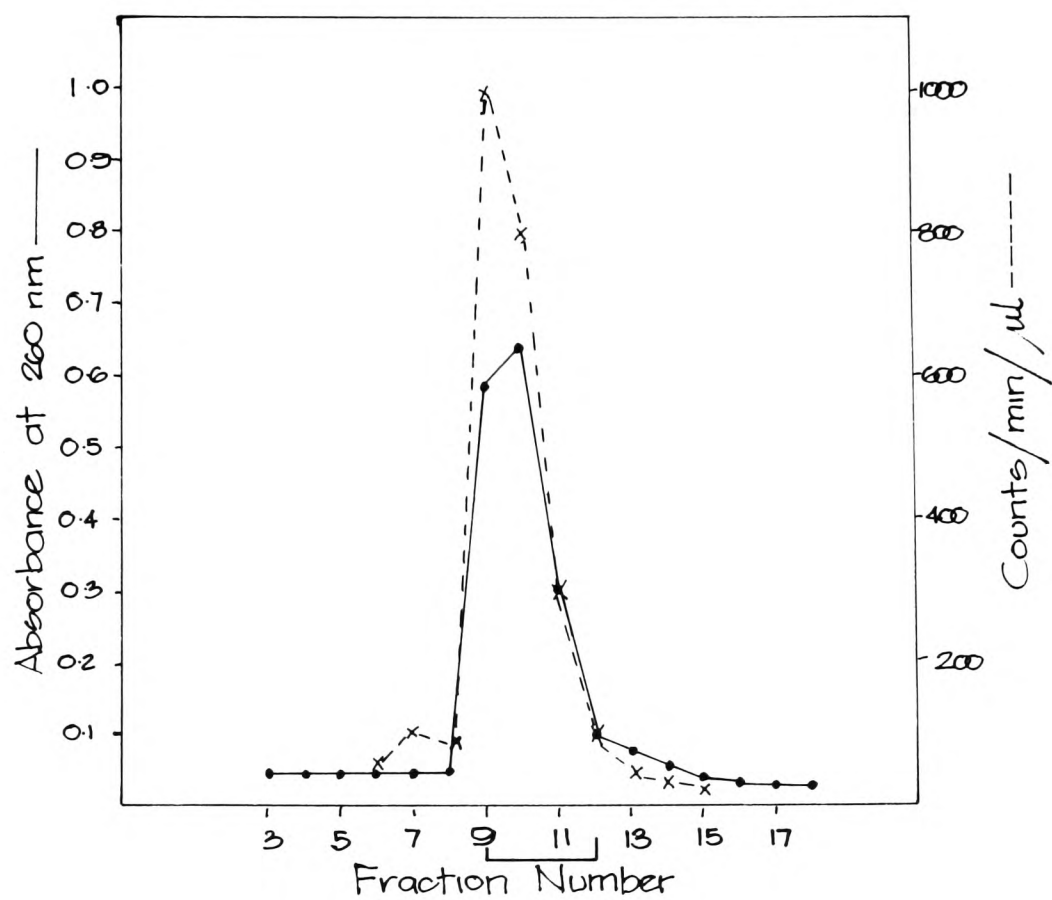


Fig. 13. The peak of radioactive satellite I crRNA eluted from a Sephadex G-50 column.

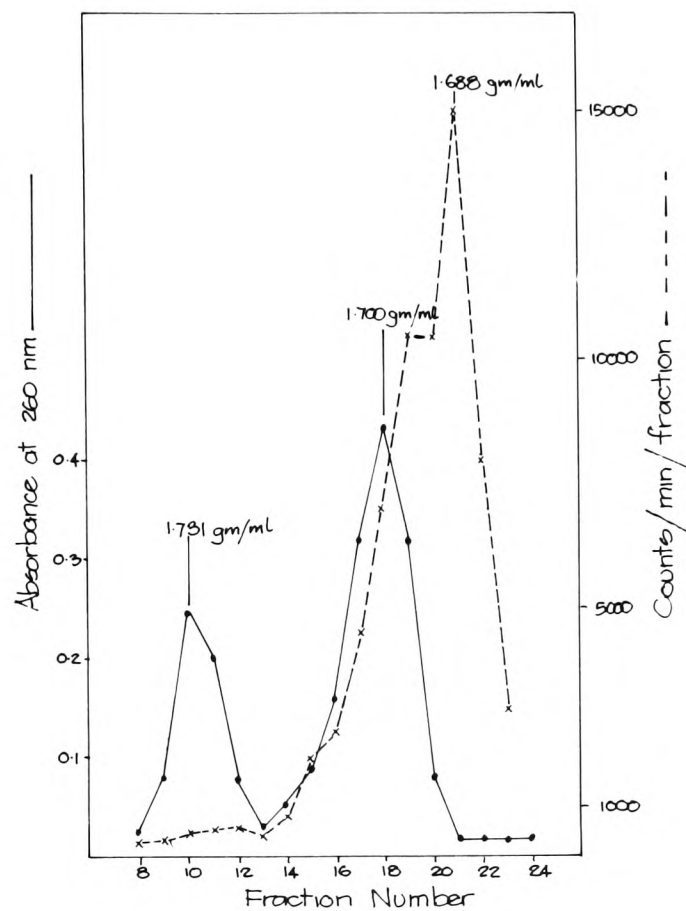


Fig. 14. Filter hybridisation result of human satellite I cRNA to human DNA fractionated in CsCl. 2×10^5 counts/min of satellite I cRNA was added to 0.7 ml of incubation mix and hybridised at 64°C overnight.

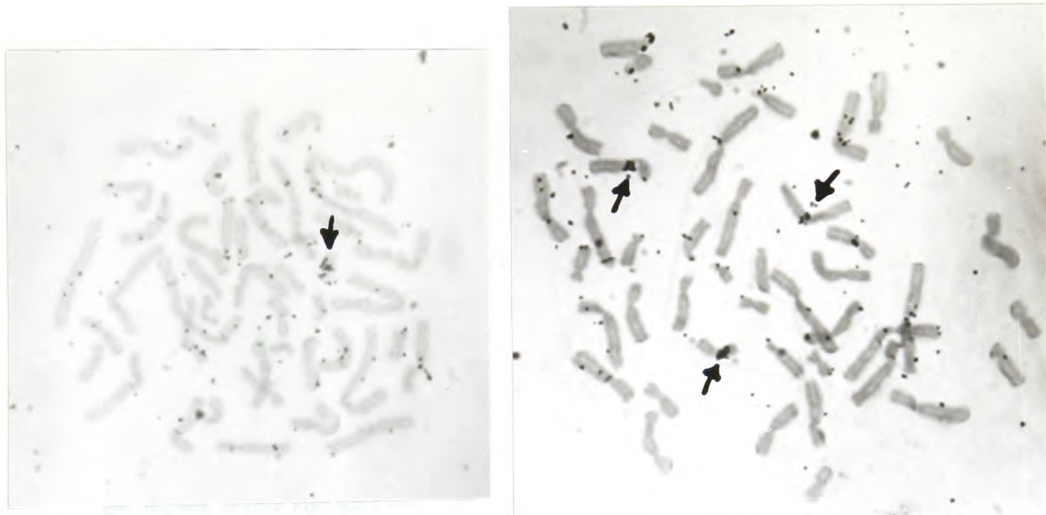


Figure 15. In situ hybridisation of satellite I cRNA to human chromosomes
 (a) to a male karyotype
 (b) to a female karyotype with two C9 chromosomes and a chromosome
 containing a centromeric section of translocated C9 hetero-
 chromatin.

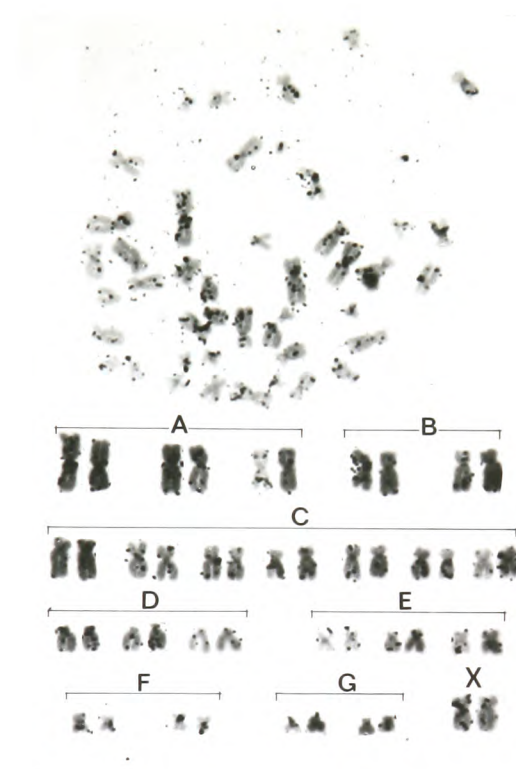


Fig. 16. In situ hybridisation of RNA complementary to Cot 10 human DNA to human metaphase chromosomes.

Comparison of the author's results with those summarised in Table 21. Satellite III

Corneo et al (1971) found that satellite III comprises 1.5% of the human genome. Assuming this to be an accurate figure, the 45 ug prepared in the present study represents 25% of the theoretical yield from the 11.2 mg of whole human DNA preparatively centrifuged. It is possible that fractionation from the top of the tube would have led to an increased yield. Since fractionation leads to a certain amount of mixing of the density gradient the nearer to the desired DNA band the fractionation begins, the less mixing will have occurred by the time the satellite is collected. Consequently it is more efficient to fractionate tubes containing light satellite DNAs from the top of the gradient. Dripping the tubes from the bottom, as was done in this and all subsequent fractionations may have led to more mixing of the equilibrium gradient before the satellite emerged from the tube than was necessary. There was certainly more DNA of buoyant density = 1.698 gm/ml present in each of the preparative gradients (Figures 1 and 2) than it was possible to isolate in pure form. It is not clear whether this was satellite III or homogeneous mainband DNA.

The buoyant density (Figure 3) and thermal denaturation data (Figure 4) for human satellite III are summarised in Table 3. The results found by the author and by Corneo et al (1971) are comparable and only minor differences were observed. There seems little doubt that the satellites are the same and this was confirmed by the identical in situ localisation found with RNA complementary to samples from each source.

Table 3. Comparison of buoyant density and thermal denaturation data for human satellite III.

| | Prosser | Corneo et al (1971) |
|--|--------------------------------------|---------------------|
| Buoyant density (in gm/ml in neutral CsCl) | | |
| Native | 1.698 | 1.696 |
| Denatured | 1.712 | 1.715 |
| Renatured | 1.705 and 1.712 | 1.703 |
| Separated single strands (in alkaline CsCl) | - | 1.740 and 1.754 |
| T _m (in 0.1 x SSC) | | |
| Native | 70° C | 69° C |
| Percent increase in hyperchromicity | 35% | - |
| Renatured (uncorrected and corrected for single strand hyperchromicity) | Uncorrected 63° C Corrected 65° C | - |
| Percent increase in hyperchromicity | 33% 24% | - |

Corneo showed a single buoyant density value for renatured satellite III while the author found that the satellite renatured to a Cot of 10^{-3} had two buoyant density peaks, that of renatured satellite and that of single stranded DNA. Corneo et al (1970a) and Mitchell (personal communication) have shown that there is a homogeneous human mainband component with buoyant density of 1.696 gm/ml. Since it is not known exactly how the metal ion: Cs_2SO_4 gradient method separates DNAs of different buoyant densities it may be that some non-satellite DNA with the same buoyant density was extracted with the satellite fraction. In the curve for the remelt shown in Figure 4 this would appear to be the case, although it is difficult to see why the "mainband" fraction was not apparent in the melting curve of native satellite illustrated in the same figure. If it is so, it would appear that any "contaminant" fractions do not affect the pattern of in situ hybridisation. The thermal denaturation profiles obtained by Corneo et al (1971) are similar to those found here. Their native melt showed a single sharp rise in hyperchromicity while the remelt showed a long foot, perhaps due to single strand hyperchromicity and a sharp rise possibly due to denaturation of the duplex molecules.

The percent GC content of human satellite III estimated on the basis of its buoyant density and T_m values is 35 to 38%, and the estimates from the two sources are comparable. The figures are as follows:

Estimation of GC content of Human satellite III

| | Buoyant density in gm/ml and Tm | Percent GC content |
|------------------------------|------------------------------------|--|
| Native (Prosser) | 1.698 | 37 (Speirs, 1972) |
| | | 36 (Sueoka <u>et al</u> 1959) |
| (Corneo <u>et al</u> , 1971) | 1.696 | 35 (Speirs, 1972) |
| | | 34 (Sueoka <u>et al</u> 1959) |
| Denatured (Prosser) | 1.712 | 34 (Sueoka <u>et al</u> 1959) |
| (Corneo) | 1.715 | 37 (Sueoka <u>et al</u> 1959) |
| Tm (Prosser) | 70°C | 38 (Marmur and Doty, 1959 and 1971) |
| (Corneo) | 69°C | 36 (Marmur and Doty 1959 and 1961) |

2. Satellite I

The result that Satellite I constitutes 0.4% of the human genome is in accordance with Corneo et al (1967) who estimated that it constituted less than 1% of the genome, and with Schildkraut and Maio (1969) who estimated that their light satellite constituted 0.2% of the HeLa genome.

The buoyant density and thermal denaturation data for human satellite I found by the author, by Corneo et al (1967, 1968a) and by Schildkraut and Maio (1969) are summarised in Table 4.

There are slight differences between the buoyant density results particularly noticeable for the separated single strands of the satellite in alkaline CsCl. However, the buoyant densities for these strands found by the author and by Schildkraut and Maio are comparable. Although the alkaline CsCl peaks of DNA are not sharp it appears that the strands of part of the satellite do separate. There is DNA material present which does not undergo strand separation and is seen to band between

Table 4. Comparison of the buoyant density and thermal denaturation data for human satellite I.

| | Prosser | Corneo et al (1968a) | Schildkraut and Maio (1969) |
|--|-------------------------------|-------------------------|--------------------------------|
| Buoyant density (in gm/ml in neutral CsCl) | | | |
| Native | 1.686 | 1.687 | 1.686 |
| Denatured | 1.699 | 1.704 | - |
| Renatured | 1.689 | 1.694 | - |
| Separated single strands (in alkaline CsCl) | 1.736 and 1.766 with 1.758 | 1.738 and 1.707 | 1.731 and 1.764 with 1.750 |
| T _m (in 0.1 x SSC) | | | |
| Native | 60°C | 65°C | - |
| Percent increase in hyperchromicity | 31% | - | - |
| Renatured | 55°C | - | - |
| Percent increase in hyperchromicity | 24% | - | - |

the light and heavy peaks. A similar but much sharper buoyant density profile was found by Schildkraut and Maio. Two peaks of equal size were found representing the light and heavy strands with a lower and broader band between them representing material that had not separated.

There is a rather large difference between the T_m for native satellite I found by the author and that found by Corneo et al (1968a). The estimation of GC content based on the T_m values (listed below) would suggest that the author's T_m is too low, although it is known that the GC content of certain low density satellites cannot be accurately estimated from their buoyant density and thermal denaturation data (Blumenfeld et al, 1973). The temperature of the melting experiment was monitored on two independent recorders, the Unicam temperature programmer (Sp876) and a thermistor made by G.H. Zeal Ltd., London.

Estimation of GC content of Human satellite I are as follows:

| | Buoyant density in gm/ml and T_m | Percent GC content |
|-------------------------------|---------------------------------------|--|
| Native (Prosser) | 1.686 | 25 (Speirs, 1972) |
| | | 24 (Sueoka <u>et al</u> 1959) |
| (Corneo <u>et al</u> , 1968a) | 1.687 | 26 (Speirs, 1972) |
| | | 25 (Sueoka <u>et al</u> 1959) |
| Denatured (Prosser) | 1.699 | 23 (Sueoka <u>et al</u> 1959) |
| (Corneo) | 1.704 | 27 (Sueoka <u>et al</u> 1959) |
| T_m (Prosser) | 60°C | 14 (Marmur and Doty, 1959 and 1961) |
| (Corneo) | 65°C | 26 (Marmur and Doty 1959 and 1961) |

Corneo et al (1967) indicated that satellite I renatured rapidly but to a limited extent. The author found it had 77% renatured at a Cot of 1 and that the buoyant density of Cot 1 renatured satellite closely approached that of native satellite DNA. Corneo's remelt of Satellite I showed a long foot, possibly due to single strand hyperchromicity, followed by a sharp rise perhaps due to denaturation of the duplex molecules, while the author found a homogeneous remelt and for this reason did not calculate the effect of single strand hyperchromicity.

3. Rapidly renaturing DNA

The random hybridisation in situ of RNA complementary to rapidly renaturing DNA was in accordance with the results of Arrighi et al (1971) who found that RNA complementary to repetitious DNA was located along the entire length of the chromosomes. Both results agreed with the data of Britten and Davidson (1969) who found that for bovine (and probably other organisms) repeated sequences were intimately interspersed with non-repeated sequences throughout the length of the genome.

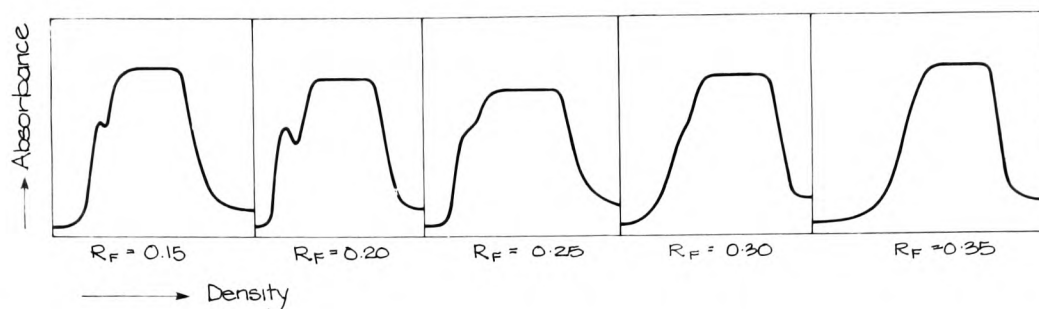


Fig. 17. Analytical equilibrium density gradient centrifugation of chimpanzee DNA (70 $\mu\text{g/ml}$) in $\text{Ag}^+ - \text{Cs}_2\text{SO}_4$ at five molar ratios. The conditions of centrifugation are described on page 65.

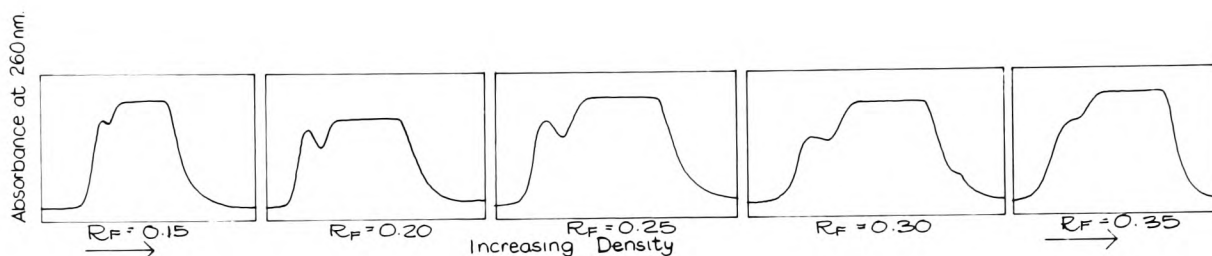


Fig. 18. Analytical equilibrium density gradient centrifugation of chimpanzee DNA (50 $\mu\text{g/ml}$) in $\text{Ag}^+ - \text{Cs}_2\text{SO}_4$ at five molar ratios. The conditions of centrifugation are described on page 66.

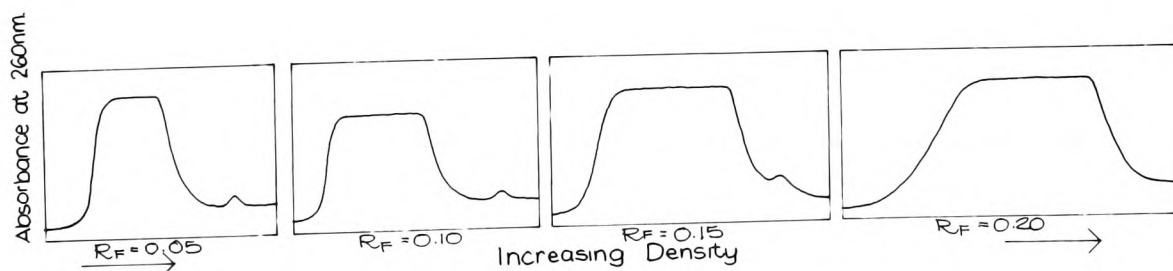


Fig. 19. Analytical equilibrium density gradient centrifugation of chimpanzee DNA (50 $\mu\text{g/ml}$) in $\text{Hg}^{++} - \text{Cs}_2\text{SO}_4$ at four molar ratios. The conditions of centrifugation are described on page 66.

SECTION B. SATELLITE DNA IN THREE OTHER PRIMATES

Introduction

The question of the species specificity of satellite DNAs is largely unresolved (see pages 11 to 14) but is of considerable interest from the standpoint of evolution. However, in the Rodentia over twenty separate species have been investigated, and homologies between satellite fractions have been reported only in some species of the genus Mus (Sutton and McCallum, 1972). In the higher primates only the human satellite DNAs have been investigated (see Table 1) and the following section describes the isolation of a number of new satellite DNAs from chimpanzee, orangutan and baboon. These new fractions enabled an investigation into the relationships between the satellite DNAs of man, chimpanzee, orangutan and baboon.

The Chimpanzee Genome

1. Analytical centrifugation

To determine if satellite fractions were present in chimpanzee DNA, samples were centrifuged in CsCl and Ag^+ - or Hg^{++} - Cs_2SO_4 density gradients in the analytical ultracentrifuge.

(a) 5 ug and 25 ug samples were centrifuged in CsCl but no satellite fractions were observed.

(b) Samples of DNA containing 70 ug/ml were centrifuged in Ag^+ - Cs_2SO_4 with molar ratios of Ag^+ :DNA-P of 0.15, 0.20, 0.25, 0.30 and 0.35 (Figure 17). A satellite fraction, visible in the first three of these samples, was most clearly separated from the mainband when a molar ratio of 0.20 was used. This satellite, called chimpanzee satellite A was isolated, characterized, transcribed and used for

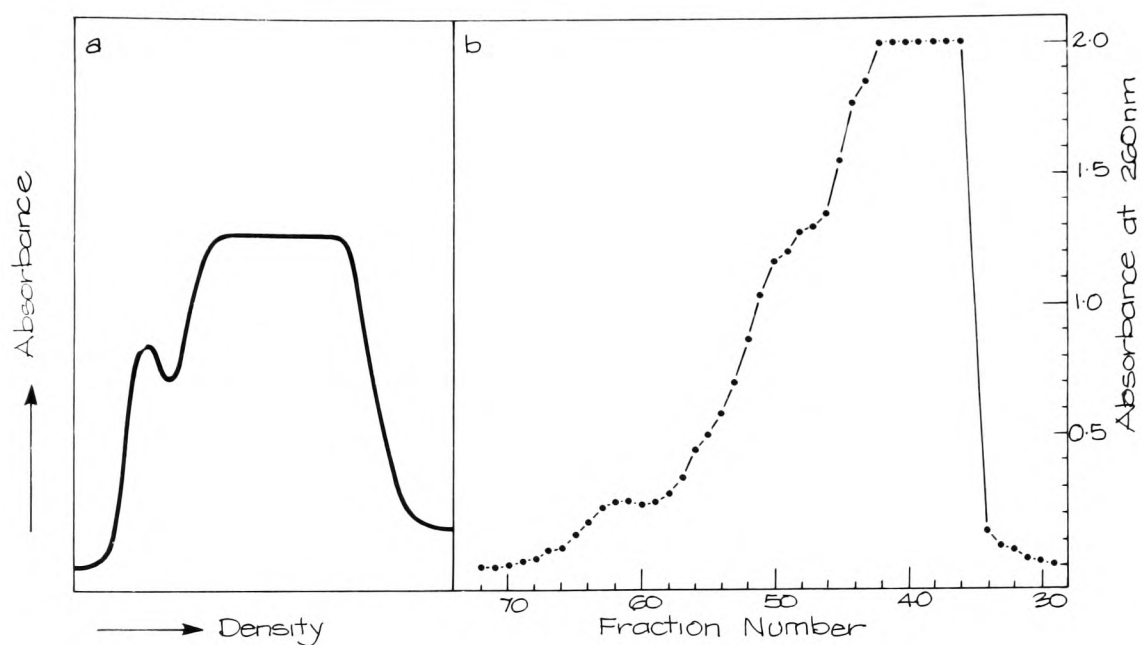


Fig. 20. Analytical (a) and preparative (b) equilibrium density gradient centrifugation of chimpanzee total DNA in $\text{Ag}^+ \text{Cs}_2\text{SO}_4$, $R_F = 0.20$. The conditions of preparation are reported on page 66. The small peak corresponds to satellite DNA, the large peak to mainband DNA.

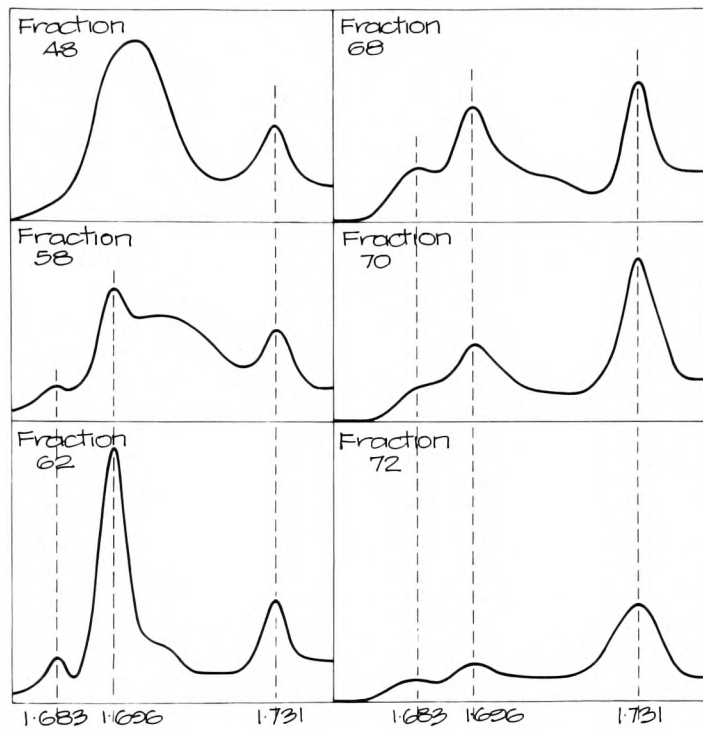


Fig. 21. Microdensitometer tracings of fractions of chimpanzee DNA (from the preparative centrifugation in $\text{Ag}^+ - \text{Cs}_2\text{SO}_4$, Fig. 20b) centrifuged in neutral CsCl in the analytical ultracentrifuge. The fraction numbers correspond to those in Fig. 20b. M. lys. DNA, buoyant density = 1.731 gm/ml was added as a marker.

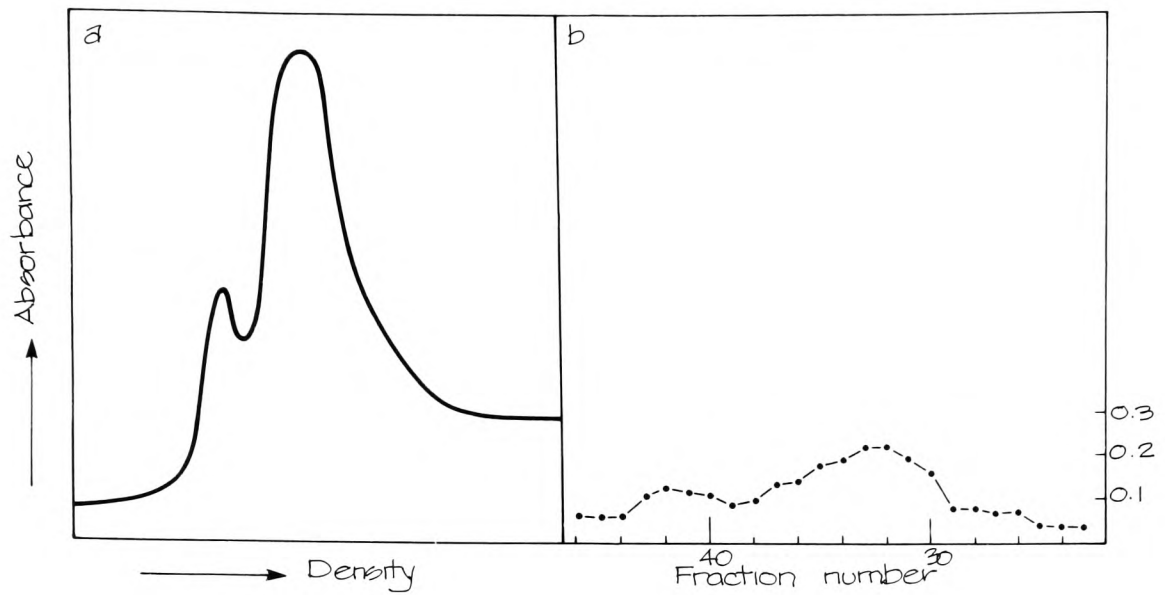


Fig. 22. Analytical (a) and preparative (b) equilibrium density gradient centrifugation of chimpanzee satellite DNA (fractions 60 - 70 of Fig. 20b) recentrifuged in CsCl as described on page 66. The small peak corresponds to satellite B, the major peak to satellite A. Mainband DNA with a density of 1.700 gm/ml is not shown.

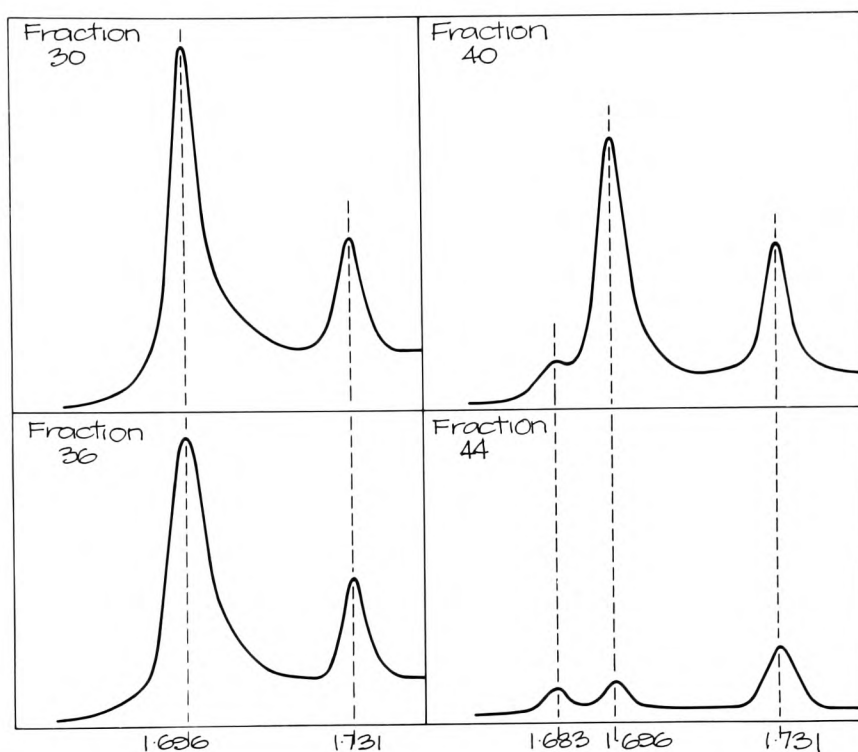


Fig. 23. Microdensitometer tracings of fractions of chimpanzee DNA (from the preparative centrifugation in CsCl, Fig. 22b) centrifuged in neutral CsCl. The fraction numbers correspond to those in Fig. 22b. M. lys. DNA, buoyant density = 1.731 gm/ml was added as a marker.

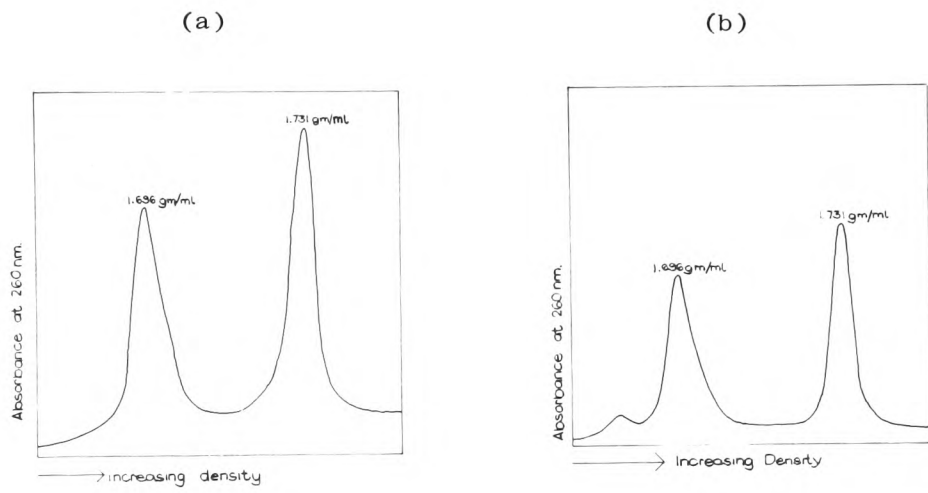


Fig. 24. Pure satellite A (a) and satellite B contaminated with satellite A (b) centrifuged to equilibrium in neutral CsCl. M. lys. DNA, buoyant density = 1.731 gm/ml was added as a marker.

hybridisation experiments.

(c) Samples of DNA containing 50 ug/ml were centrifuged in $\text{Ag}^+ - \text{Cs}_2\text{SO}_4$ using the above molar ratios (Figure 18). The microdensitometer profiles were similar to those in which 70 ug/ml were used except that the large light satellite fraction was most clearly separated from the mainband in the sample containing a molar ratio of 0.25 and an additional heavy satellite was visible in the sample containing a molar ratio of 0.30. On the microdensitometer tracing this would be called a shoulder but it is clearly a satellite DNA in the analytical negative. This fraction was not isolated.

(d) Samples of DNA containing 50 ug/ml were centrifuged in $\text{Hg}^{++} - \text{Cs}_2\text{SO}_4$ with molar ratios of $\text{Hg}^{++}:\text{DNA-P}$ of 0.05, 0.10, 0.15 and 0.20 (Figure 19). A small heavy satellite, visible in the first three of these samples, was most clearly separated from the mainband in the sample using a molar ratio of 0.05. This satellite, called chimpanzee satellite B, was isolated but at the time of writing has not been characterized.

2. Satellite A

a. Preparation

Preparative centrifugation was carried out using a molar ratio of $\text{Ag}^+:\text{DNA-P}$ of 0.20. Eight tubes containing 20 ml/tube were centrifuged in the 8 x 50 rotor of the MSE ultracentrifuge. 6-drop fractions were collected, diluted and their absorbance at 260 nm determined. Figure 20 shows the OD profile of a fractionated preparative gradient together with a microdensitometer tracing of a sample from the preparation analyzed in the Model E. Figure 21 shows the microdensitometer tracings of selected fractions from the preparative gradient analyzed in the Model E. Fractions 60 to 70 from Figure 24 together with analogous fractions from the remaining tubes were pooled, concentrated,

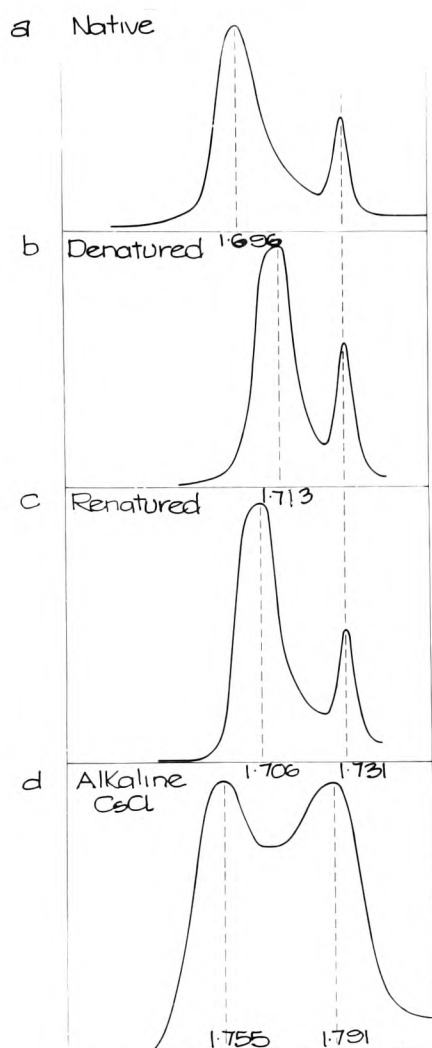


Fig. 25. Microdensitometer tracings of chimpanzee satellite A centrifuged to equilibrium in the analytical ultracentrifuge in neutral CsCl (a) native (b) denatured by heating at 100°C for 10 minutes in 0.1 x SSC followed by rapid cooling (c) renatured at 10 μ g/ml at 65°C for 5 hours in 2 x SSC after heat denaturation as in (b). Separation of strands in alkaline CsCl is shown in (d). Densities in neutral CsCl were determined from M. lys. DNA, buoyant density = 1.731 gm/ml and in alkaline CsCl from the limiting isoconcentration distance and the initial density of the solution.

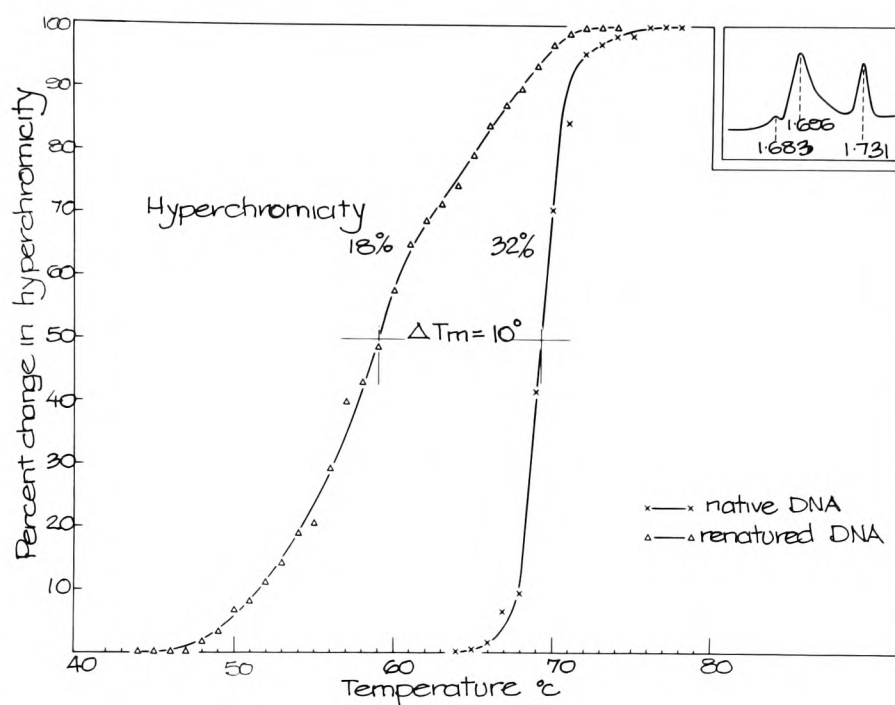


Fig. 26. The melting curves of native and renatured chimpanzee satellite A in 0.1 x SSC. The renatured satellite was first denatured in 0.1 x SSC at 100°C for 10 minutes, then made up to 2 x SSC and renatured for 5 hours at 20 μ g/ml at 65°C. It was dialyzed into 0.1 x SSC before melting. The ΔT_m between native and renatured satellite is 10° (uncorrected for single strand hyperchromicity) or 9° (corrected).

dialyzed into 0.1 x SSC and centrifuged in CsCl in the 10 x 10 rotor of the MSE. 4-drop fractions were collected, diluted and their absorbance at 260 nm determined. Figure 22 shows this result together with a microdensitometer tracing of a sample from the preparation analyzed in the Model E. The single satellite peak seen in the $\text{Ag}^+ - \text{Cs}_2\text{SO}_4$ gradient is observed to break into two distinct satellites in CsCl centrifugation, satellite A with a buoyant density of 1.696 gm/ml and satellite B with a buoyant density of 1.683 gm/ml. Chimpanzee mainband DNA has a buoyant density of 1.700 gm/ml. Figure 23 shows microdensitometer tracings of selected fractions from the CsCl gradient. 85 ug of pure satellite A was isolated from both the $\text{Ag}^+ - \text{Cs}_2\text{SO}_4$ and the CsCl gradients, a yield of less than 1% of the total DNA centrifuged (Figure 24a). Satellite B was not prepared free from Satellite A contamination by this method (Figure 24b).

b. Characteristics

The characteristic buoyant densities of chimpanzee satellite A in neutral CsCl were found to be

| | | |
|-------------|--------------------------|--------------|
| 1.696 gm/ml | native | (Figure 25a) |
| 1.713 gm/ml | denatured | (Figure 25b) |
| 1.706 gm/ml | renatured to a Cot of 5G | (Figure 25c) |

15 ug of satellite A were centrifuged in alkaline CsCl and the denatured satellite was observed to separate into a light and a heavy strand with buoyant densities of 1.755 gm/ml and 1.791 gm/ml respectively (Figure 25d).

Thermal denaturation profiles were obtained for native satellite A and satellite denatured and renatured to a Cot of 1 (as described for human satellite III). 10 ug of the satellite was melted in the Unicam spectrophotometer. Native satellite A has a T_m of 69°C and

shows 32% increase in hyperchromicity. Cot 1 satellite A has a T_m of 59°C and shows an 18% increase in hyperchromicity. The results are plotted in Figure 26 and show a ΔT_m of 10° . If the effect of single strand hyperchromicity is taken into consideration when viewing the melting curve of renatured chimpanzee satellite A, a slightly different interpretation is afforded. This can be done in the following way. From the equation $32a + 5.3(1-a) = 18$, where a is the proportion of DNA in duplex form, 32 is the percent hyperchromicity obtained for native duplex chimpanzee satellite A, 5.3 is the percent hyperchromicity above 45°C for single stranded DNA (Bishop and Pemberton, 1972) and 18 is the percent hyperchromicity for "renatured" chimpanzee satellite DNA containing a mixture of single stranded and duplex molecules, it is calculated that 15.5% of the hyperchromicity indicated on the graph is due to single stranded hyperchromicity. The T_m calculated in this way is 60°C , giving a ΔT_m of 9° . The biphasic character of the renatured melt indicates that two populations of molecules are present, one more homogeneous than the other.

The percent GC content of chimpanzee satellite A estimated on the basis of its buoyant density and T_m values is 34 to 36%.

| Chimpanzee satellite A | | |
|------------------------|----------------------|-------------------------------------|
| | Buoyant density | Percent GC content |
| Native | 1.696 | 35 (Speirs, 1972) |
| | | 33 (Sueoka, <u>et al</u> 1959) |
| Denatured | 1.713 | 35 (Sueoka, <u>et al</u> 1959) |
| T_m | 69°C | 36 (Marmur and Doty, 1959 and 1961) |

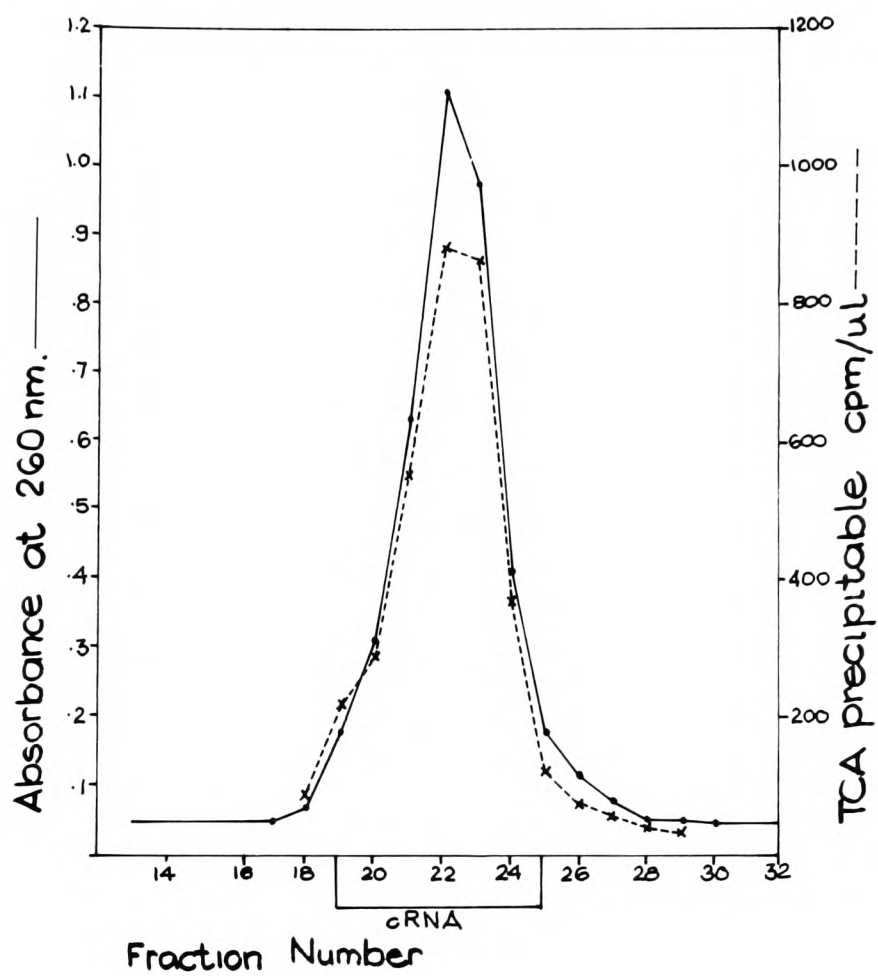


Fig. 27. The peak of radioactive satellite A crRNA from a Sephadex G-50 column.

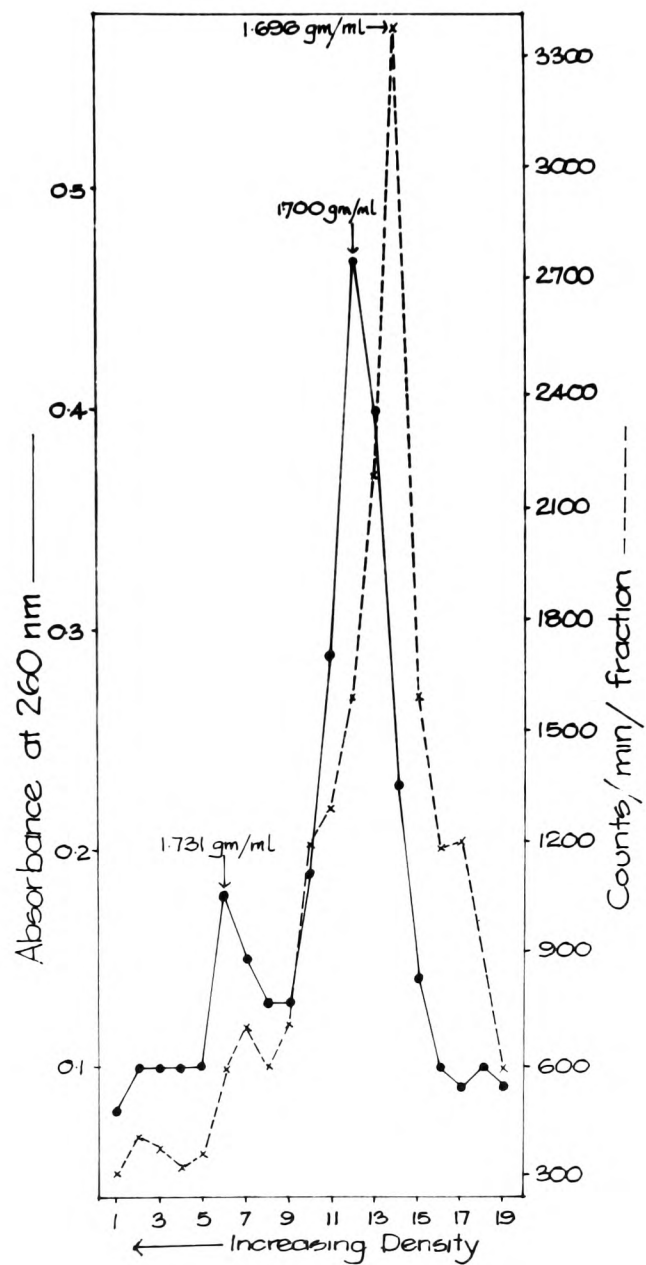


Fig. 28. Filter hybridisation result of chimpanzee satellite A crRNA to chimpanzee DNA fractionated in CsCl. $1.1\text{--}1.2 \times 10^5$ counts/min of satellite A crRNA was added to 0.5 ml of incubation mix and hybridisation took place at 68°C for 4 hours. The hybridisation was carried out by M. Moar.

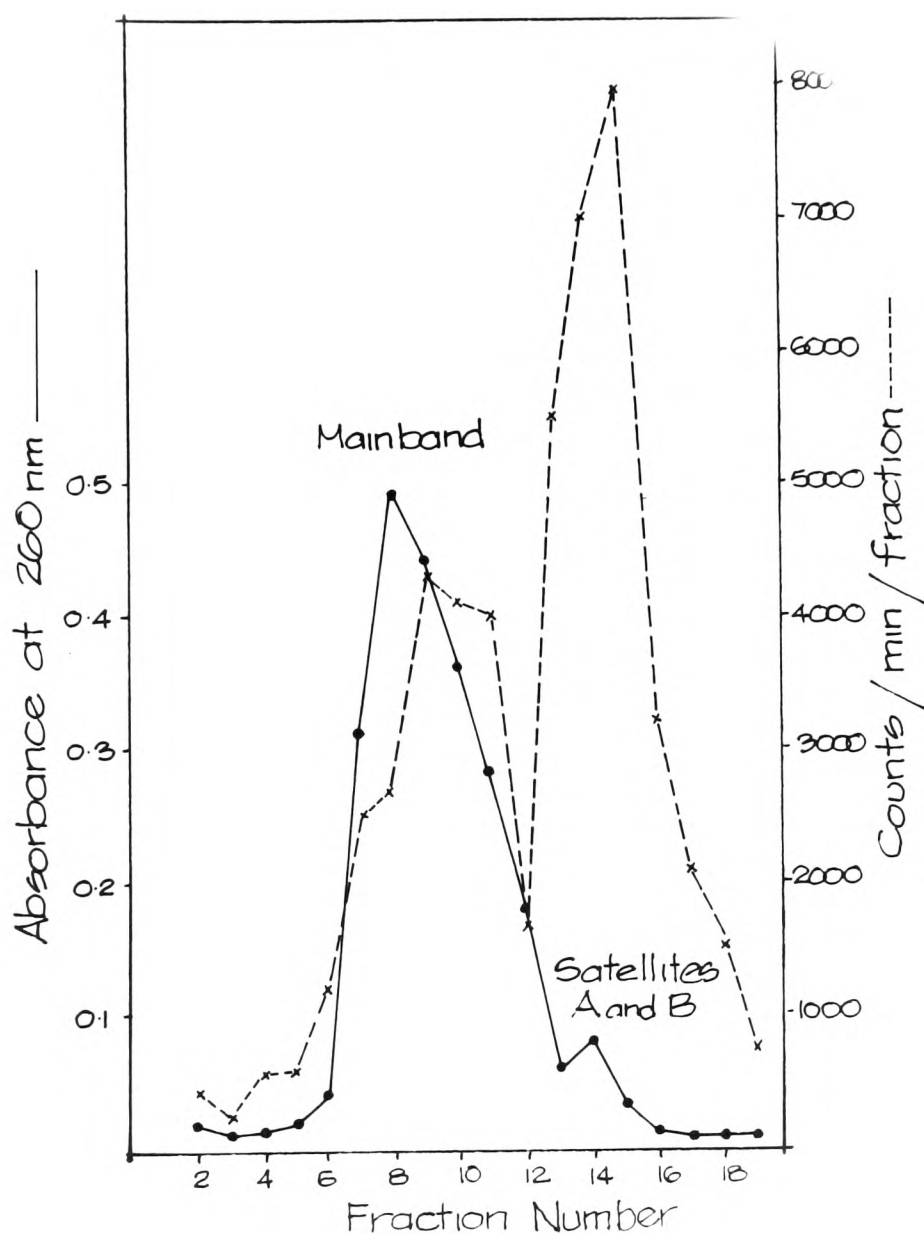


Fig. 29. Filter hybridisation result of chimpanzee satellite A cRNA to chimpanzee DNA fractionated in $\text{Ag}^+ - \text{Cs}_2\text{SO}_4$, $R_F = 0.20$. 2.1×10^5 counts/min of satellite A cRNA was added to 0.7 ml of incubation mix and hybridisation took place in 65°C overnight.



Fig. 30. In situ hybridisation of chimpanzee satellite A cRNA to chimpanzee metaphase chromosomes.

c. Transcription

20 ug of satellite A were transcribed using all four nucleotides radioactive. Figure 27 shows the peak of RNA eluted from the Sephadex G-50 column. Fractions 19-25 were pooled, lyophilised and taken up in distilled water to make 0.15 ml of chimpanzee satellite A crRNA in 4 x SSC with a specific activity of 1.1×10^8 dpm/ug. The crRNA contained 4.5×10^4 cpm/ul.

d. Gradient hybridisation

Chimpanzee satellite A crRNA was hybridised to chimpanzee and rat DNA fractionated in Cs salt density gradients. The composition of the gradients and hybridisation details are indicated in the figure legends. Satellite A crRNA hybridised to a fraction of chimpanzee DNA having a buoyant density of $1.696 \text{ gm/ml} \pm .002 \text{ gm/ml}$ the buoyant density of the original template satellite (Figure 28). Hybridisation to control rat DNA from a CsCl gradient did not occur above background counts and is not illustrated. Satellite A crRNA was hybridised to chimpanzee DNA from a $\text{Ag}^+ - \text{Cs}_2\text{SO}_4$ gradient and was found to hybridise to the satellite A fraction, although the peak of radioactive counts per minute of the hybridised crRNA was situated to the left of the OD peak of the satellite DNA (Figure 29). The original template DNA was obtained from the light side of the satellite peak, but this unexpected hybridisation result suggests as does the melting data that chimpanzee satellite A may not be a homogeneous population of molecules. Hybridisation of satellite A crRNA also occurred to the DNA in the "mainband" peak. Experiments are under way to determine if this is real hybridisation or non-specific sticking of the crRNA to large amounts of DNA on the filters.

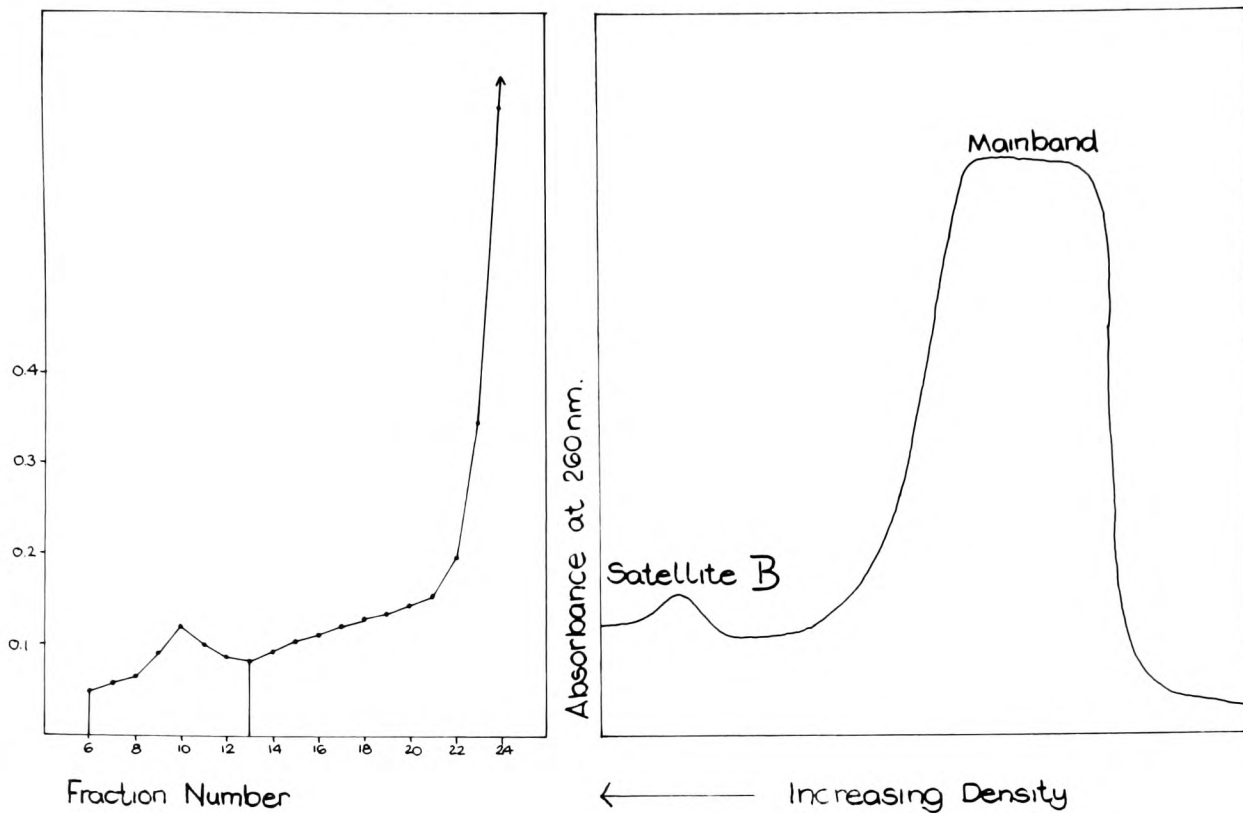


Fig. 31. Preparative (a) and analytical (b) equilibrium density gradient centrifugation of chimpanzee total DNA in $\text{Hg}^{++}\text{-Cs}_2\text{SO}_4$ $R_F = 0.05$. The conditions of preparation are reported on page 70. The small peak on the heavy side of the gradient corresponds to satellite B.

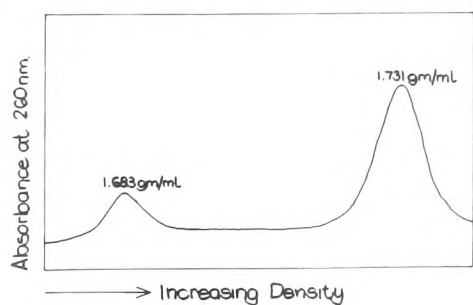


Fig. 32. Pure satellite B centrifuged to equilibrium in neutral CsCl . M. lys. DNA, buoyant density - 1.731 gm/ml was added as a marker.

e. In situ hybridisation

Chimpanzee satellite A cRNA was hybridised to chimpanzee and control chromosomes as described on page 43. Satellite A cRNA was found localised in the centromeric heterochromatin of 8 or 9 pairs of chromosomes (Figure 30). The significance of these results will be discussed more fully in the discussion section.

3. Satellite B

a. Preparation

Pure chimpanzee satellite B was prepared using a $\text{Hg}^{++}\text{-Cs}_2\text{SO}_4$ gradient of 50 μg DNA/ml and a molar ratio of Hg^{++} :DNA-P of 0.05. Four tubes containing 20 ml/tube were centrifuged in the 8 x 50 rotor of the MSE 50 ultracentrifuge. 6-drop fractions were collected, diluted and their absorbance at 260 nm determined. Figure 31 shows the OD profile of the fractionation of one tube together with a microdensitometer tracing of a sample from the same preparation analyzed in the Model E. Fractions pertaining to the satellite were dialyzed to remove the Hg^{++} ions, concentrated and dialyzed into 0.1 x SSC. The satellite was found to have a buoyant density of 1.683 gm/ml (Figure 32) in neutral CsCl which was identical to the satellite B fraction visible in the $\text{Ag}^+\text{-Cs}_2\text{SO}_4$ gradient used to prepare satellite A. 30 μg of pure satellite B was prepared in this way, a yield which constitutes slightly less than 1% of the genome. Since chimpanzee satellite B is well removed from the mainband DNA in this preparation, 1% is probably a reasonable estimate of the proportion of the genome which it constitutes.

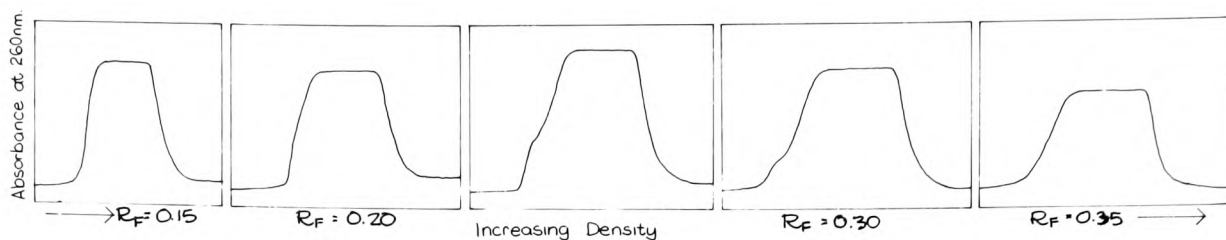


Fig. 33. Analytical equilibrium density gradient centrifugation of orangutan DNA (70 ug/ml) in $\text{Ag}^+\text{Cs}_2\text{SO}_4$ at five molar ratios. The conditions of centrifugation are described on page 71.

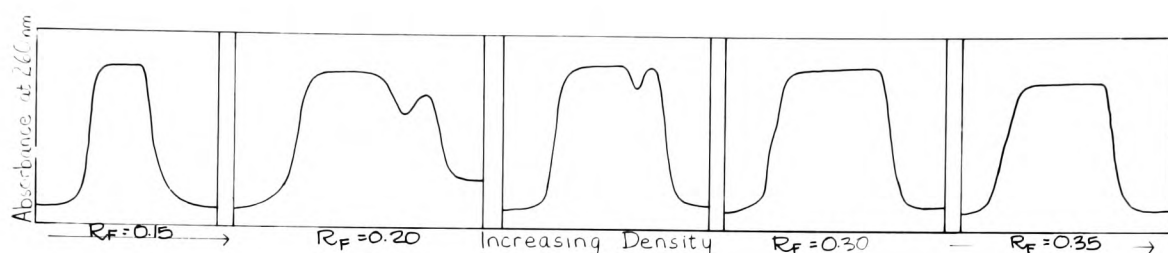


Fig. 34. Analytical equilibrium density gradient centrifugation of baboon DNA (70 ug/ml) in $\text{Ag}^+\text{Cs}_2\text{SO}_4$ at five molar ratios. The conditions of centrifugation are described on page 72.

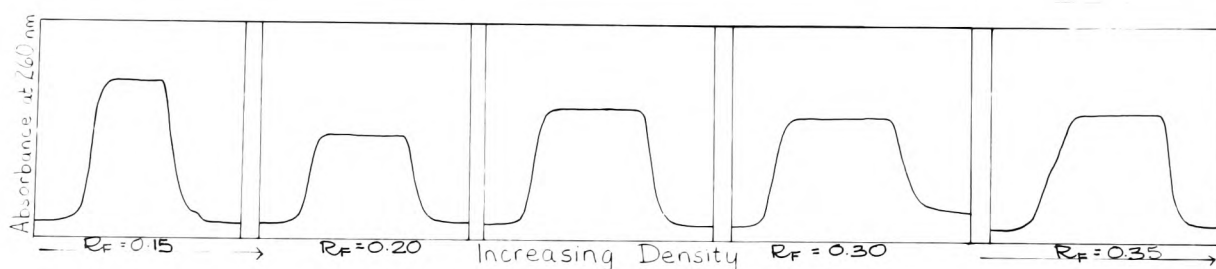


Fig. 35. Analytical equilibrium density gradient centrifugation of baboon DNA (50 ug/ml) in $\text{Ag}^+\text{Cs}_2\text{SO}_4$ at five molar ratios. The conditions of centrifugation are described on page 72.

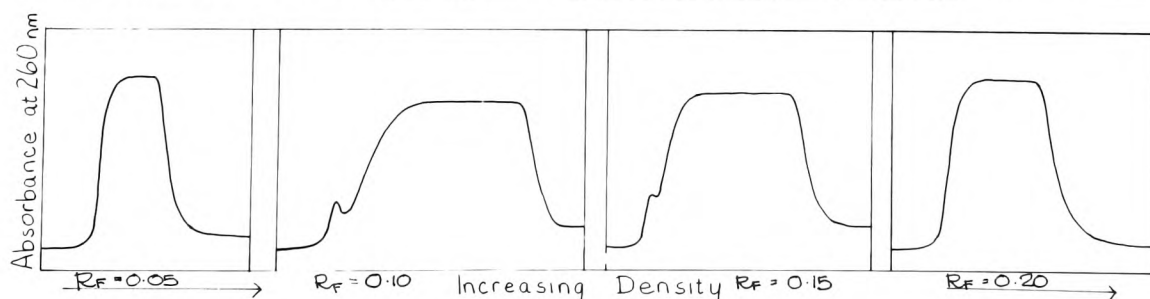


Fig. 36. Analytical equilibrium density gradient centrifugation of baboon DNA (50 ug/ml) in $\text{Hg}^{++}\text{Cs}_2\text{SO}_4$ at four molar ratios. The conditions of centrifugation are described on page 72.

The Orangutan Genome

Because of the successful cross hybridisation between human, chimpanzee and orangutan DNAs described in Section C an attempt was made to isolate orangutan satellite DNA when material became available as a gift from Dr Kurt Benirschke, the San Diego Zoo.

A poor yield of low molecular weight DNA was obtained from the frozen orangutan placenta, perhaps because DNA degradation had occurred before it was possible to freeze the tissue. This yield was separated into a high molecular weight (spoolable after alcohol precipitation) and a low molecular weight fraction (precipitated from solution by centrifugation at 10 K rpm in the Sorvall). The molecular weight of the spoolable DNA was 5×10^6 daltons which is an order of magnitude lower than that obtained for standard DNA preparations. The spoolable DNA was used for density gradient centrifugation, but because of its relatively low molecular weight, it produced fuzzy bands when centrifuged in CsCl in the Model E and no satellites were visible. $\text{Ag}^+ - \text{Cs}_2\text{SO}_4$ centrifugation was carried out using 70 ug of DNA/ml and a series of molar ratios of $\text{Ag}^+ : \text{DNA-P}$ of 0.15, 0.20, 0.25, 0.30 and 0.35 (Figure 33). A molar ratio of 0.25 produced a very broad (fuzzy) shoulder on the light side of the mainband which was less obvious when molar ratios of 0.20 and 0.30 were used. An attempt was made to isolate this light shoulder but a clean separation from mainband was not achieved.

The Baboon Genome

Baboon tissue was readily available in Edinburgh and was therefore selected as representative of a family of apes more distantly related to man than chimpanzee, orangutan or gorilla (Napier and Napier, 1967).

1. Analytical centrifugation

To determine if satellite fractions were present in baboon DNA, samples were centrifuged in CsCl and Ag^+ -or Hg^{++} - Cs_2SO_4 density gradients in the Model E analytical ultracentrifuge.

(a) 5 ug and 25 ug samples were centrifuged in neutral CsCl but no satellite fractions were observed.

(b) Samples of DNA containing 70 ug/ml were centrifuged in Ag^+ - Cs_2SO_4 with molar ratios of $\text{Ag}^+:\text{DNA-P}$ of 0.15, 0.20, 0.25, 0.30 and 0.35 (Figure 34). A large heavy satellite visible in the second and third of these samples was most clearly separated from the mainband when a molar ratio of 0.20 was used. This fraction was isolated and called baboon satellite DNA. A light shoulder was visible in the last two samples but no attempt was made to isolate it.

(c) Samples of DNA containing 50 ug/ml were centrifuged in Ag^+ - Cs_2SO_4 using the same molar ratios as in (b) above (Figure 35). No satellites were visible in any of the samples but a very small heavy shoulder was present when a molar ratio of 0.15 was used and a larger light shoulder was visible when a molar ratio of 0.35 was used. These fractions were not isolated.

(d) Samples of DNA containing 50 ug/ml were centrifuged in Hg^{++} - Cs_2SO_4 with molar ratios of $\text{Hg}^{++}:\text{DNA-P}$ of 0.05, 0.10, 0.15 and 0.20 (Figure 36). A small heavy satellite was visible in the second and third of these samples and was most clearly separated from the mainband when a molar ratio of 0.10 was used. This satellite was not isolated.

2. Baboon satellite DNA

a. Preparation

Baboon satellite DNA was prepared in Ag^+ - Cs_2SO_4 using 70 ug DNA/ml

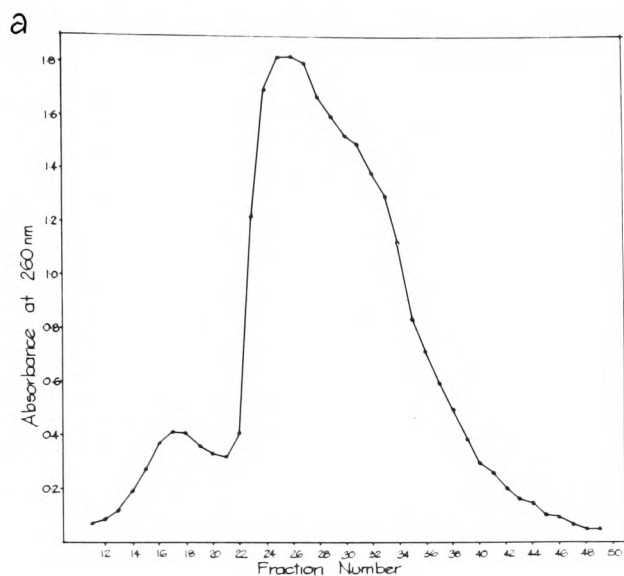


Fig. 37. Preparative (a) and analytical (b) equilibrium density gradient centrifugation of baboon total DNA in $\text{Ag}^+ - \text{Cs}_2\text{SO}_4$ $R_F = 0.20$. The conditions of preparation are reported on page 72.

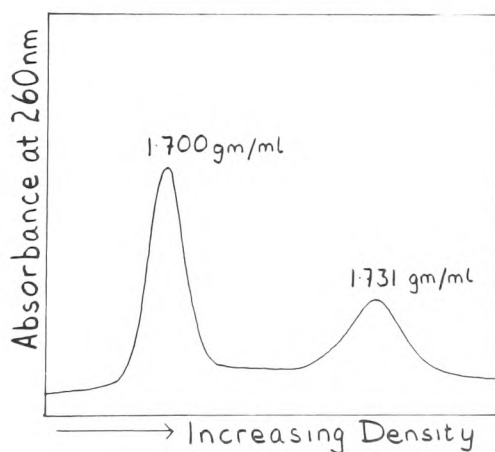
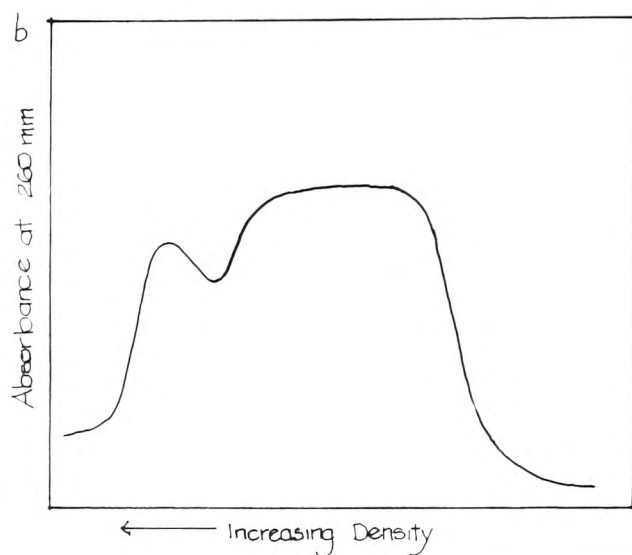


Fig. 38. Pure baboon satellite DNA centrifuged to equilibrium in neutral CsCl . M. lys. DNA, buoyant density = 1.731 gm/ml was added as a marker.

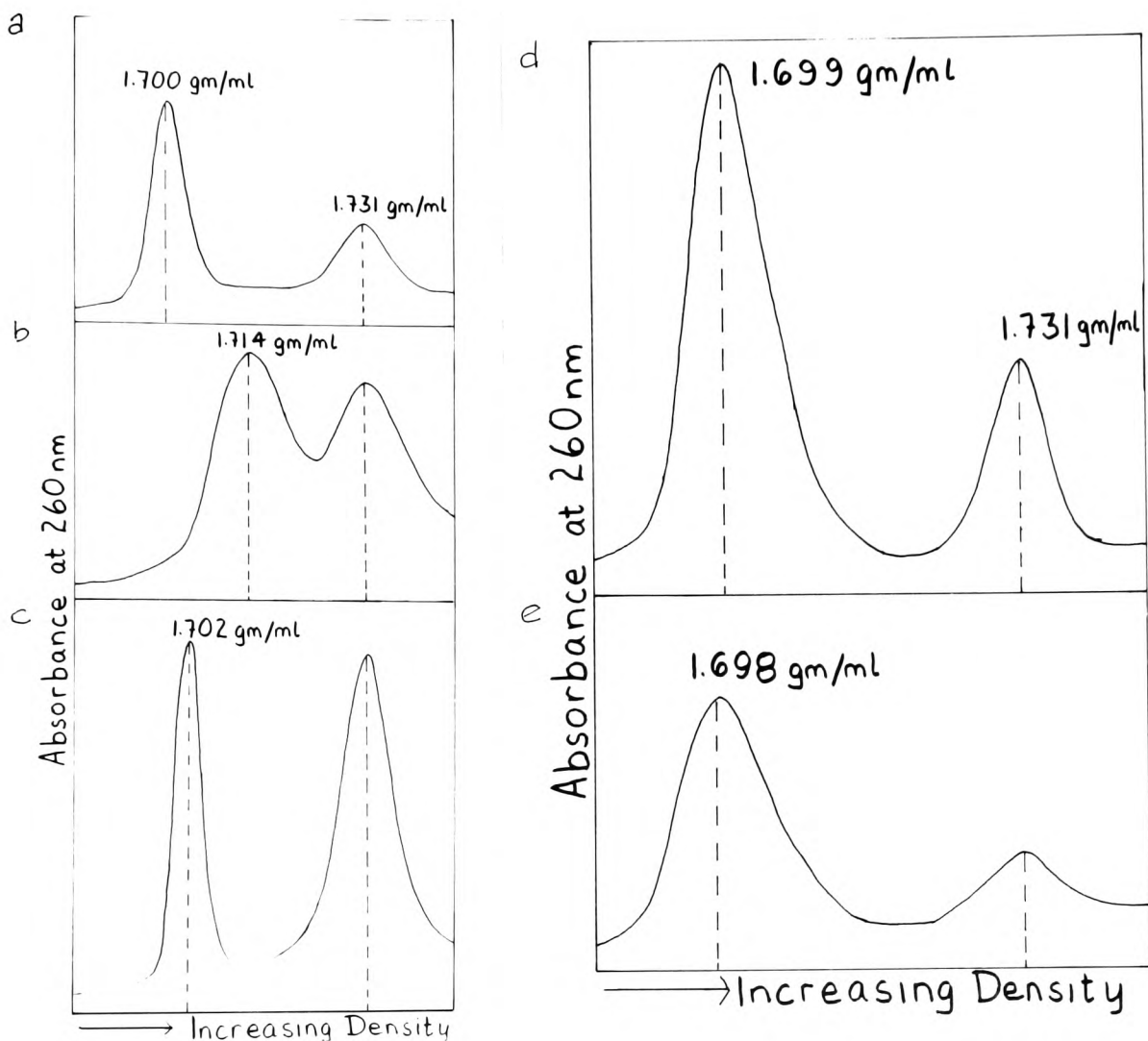


Fig. 39. Microdensitometer tracings of baboon satellite DNA centrifuged to equilibrium in the analytical ultracentrifuge in neutral CsCl (a) native (b) denatured by heating at 100°C for 10 min. in 0.1 x SSC followed by rapid cooling (c) renatured at 2 μ g/ml at 65° for 3 min. in 2 x SSC after heat denaturation in (b). Microdensitometer tracings of (d) baboon whole DNA and (e) baboon mainband DNA centrifuged to equilibrium in neutral CsCl. M. lys. DNA buoyant density = 1.731 gm/ml was added as a marker.

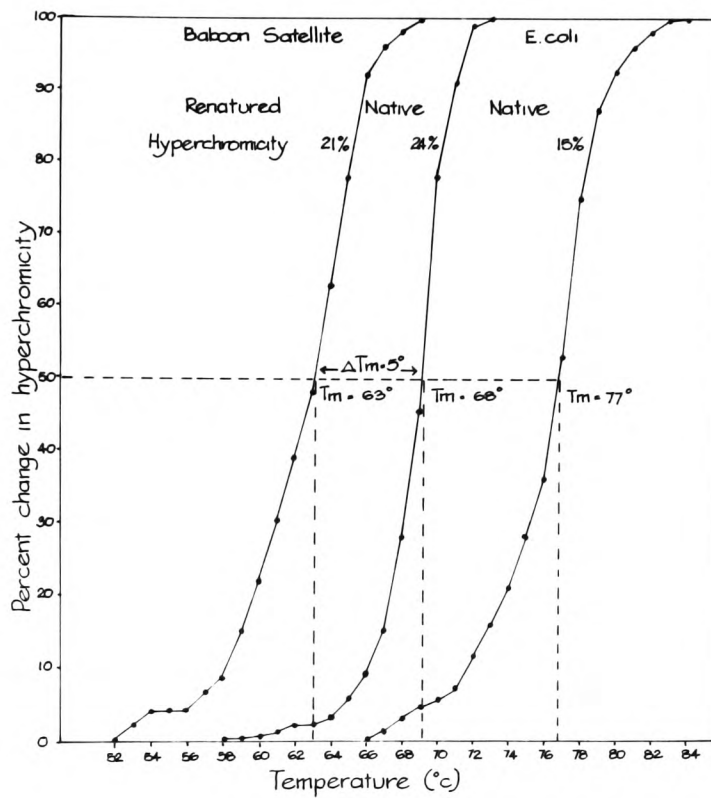


Fig. 40. The melting curves of native and renatured baboon satellite DNA in 0.1 x SSC using native E.coli DNA as a standard. The renatured sample was first denatured in 0.1 x SSC at 100°C for 10 min. then made up to 2 x SSC and renatured for 3 min. at 2 µg/ml at 66°C. It was dialyzed into 0.1 x SSC and concentrated before melting. The ΔT_m between native and renatured baboon satellite DNA is 5° (uncorrected for single strand hyperchromicity) and 4.5° (corrected).

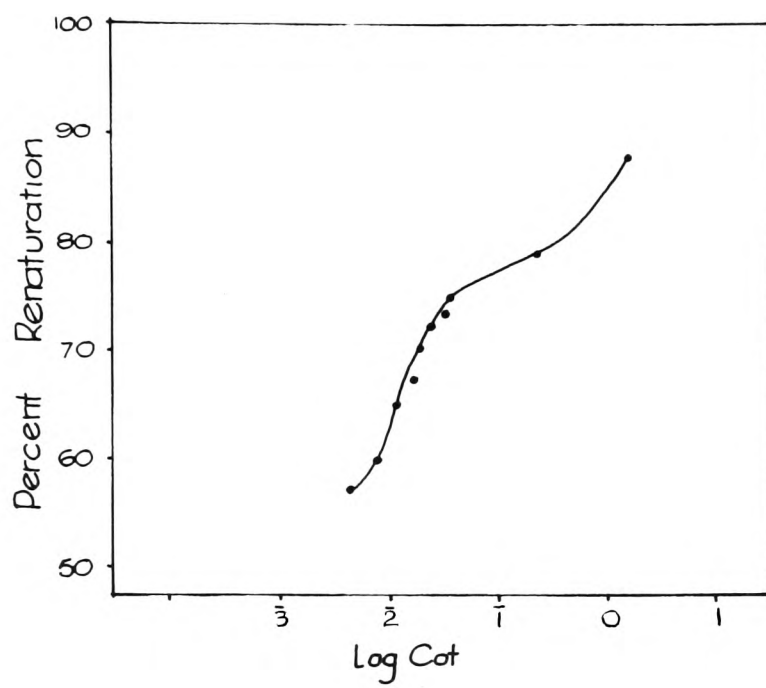


Fig. 41. Renaturation curve of baboon satellite DNA in 2 x SSC/50% formamide, showing that baboon satellite has a low $Cot_{\frac{1}{2}}$ value. Conditions for thermal renaturation are described on page 38.

and a molar ratio of 0.20. Four tubes, each containing 15 ml of solution were centrifuged in the MSE 50 ultracentrifuge. 10-drop fractions (approximately 0.40 ml) were collected, diluted and their absorbance at 260 nm determined in the Unicam spectrophotometer. Figure 37 shows the fractionation of one of the 4 tubes and a microdensitometer tracing of a sample from the preparation analyzed in the Model E. Fractions 12 to 18, which are well removed from the mainband DNA, and similar fractions from the remaining tubes were pooled, dialyzed to remove Ag^+ ions and dialyzed into 0.1 x SSC. The satellite DNA was concentrated and dialyzed into 0.1 x SSC. Fractions 19 to 22 and analogous fractions from the remaining tubes were treated in the same way and stored for subsequent removal of mainband contamination by centrifugation in CsCl. 40 ug of baboon satellite DNA was prepared in this way (Figure 38), a yield of approximately 1%. Since only those fractions of satellite DNA which were well removed from the mainband were used to prepare baboon satellite, the 1% yield is not an estimate of the total amount of this satellite found in the baboon genome.

b. Characteristics

The characteristic buoyant densities of baboon satellite DNA in neutral CsCl are

| | |
|---|--------------|
| 1.700 gm/ml native | (Figure 39a) |
| 1.714 gm/ml denatured | (Figure 39b) |
| 1.702 gm/ml renatured to a Cot of 10^{-3} | (Figure 39c) |

Baboon satellite was not centrifuged in alkaline CsCl due to scarcity of material. Baboon whole DNA centrifuged in neutral CsCl has a buoyant density of 1.699 gm/ml (Figure 39d) and baboon mainband DNA has a buoyant density of 1.698 gm/ml (Figure 39e).

Thermal denaturation profiles were obtained for native baboon satellite and satellite denatured and renatured to a Cot of 10^{-3} (as described for human satellite III). 17 μ g of the satellite was melted in $0.1 \times SSC$ in the Unicam spectrophotometer. Native satellite has a T_m of $68^\circ C$ and shows a 24% increase in hyperchromicity. Cot 10^{-3} satellite has a T_m of $63^\circ C$ and shows a 21% increase in hyperchromicity. Plotted as indicated in Figure 40 a ΔT_m of 5° is obtained. However, if the effect of single strand hyperchromicity is taken into consideration, and the calculations described for chimpanzee satellite A are carried out, it is estimated that 16% of the increase in OD shows in the remelt is due to single strand hyperchromicity. The T_m of the Cot 10^{-3} satellite calculated in this way is then $63.5^\circ C$ giving a ΔT_m of 4.5° .

The percent GC content of baboon satellite DNA based on its buoyant density and T_m values is 34-39%. The figures are as follows:

| Buoyant density in gm/ml and T_m | | |
|---------------------------------------|--------------|-------------------------------------|
| Native | 1.700 | 39 (Speirs, 1972) |
| | | 37 (Sueoka <u>et al</u> , 1959) |
| Denatured | 1.714 | 36 (Sueoka <u>et al</u> , 1959) |
| T_m | $68^\circ C$ | 34 (Marmur and Doty, 1959, 1961) |

Renaturation of baboon satellite DNA was carried out in $2 \times SSC/50\%$ formamide. There was sufficient material only for one pilot experiment and the result (Figure 41) showed that over 50% renaturation had occurred in the first 75 seconds

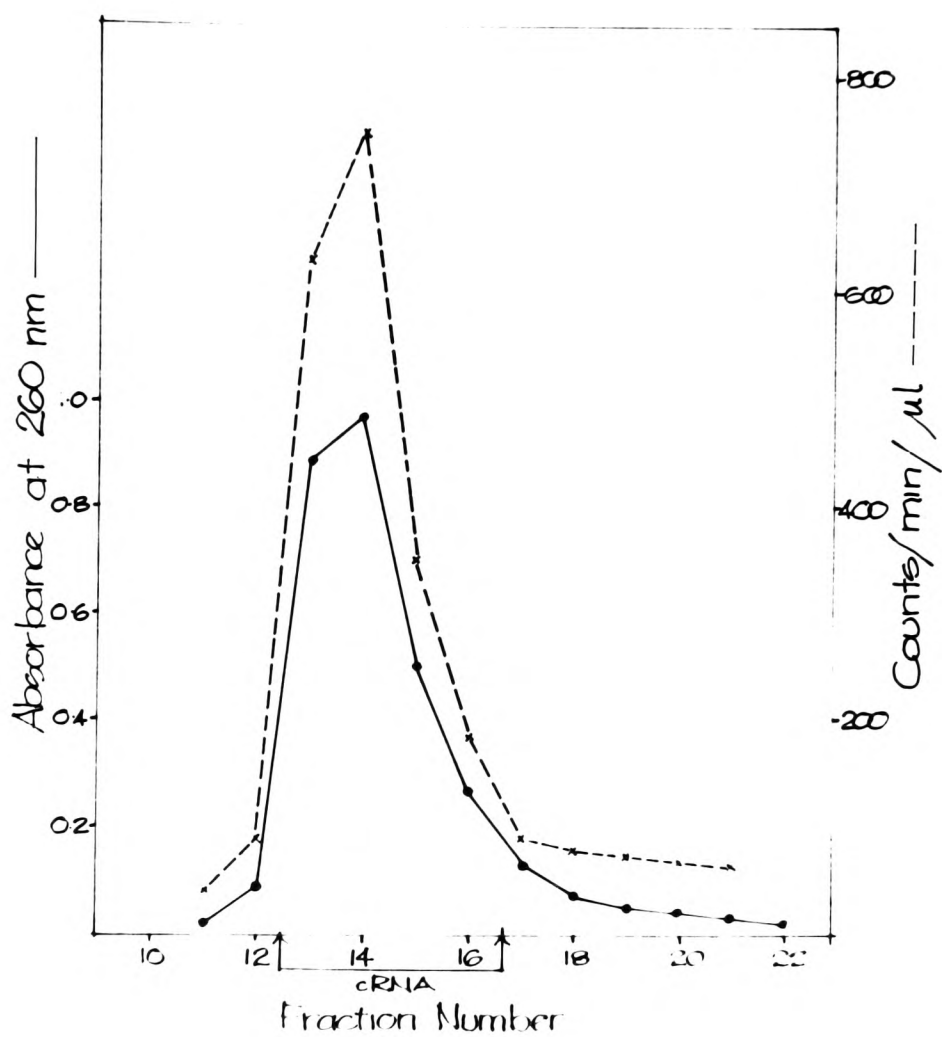


Fig. 42. The peak of radioactive baboon satellite cRNA eluted from a Sephadex G-50 column.

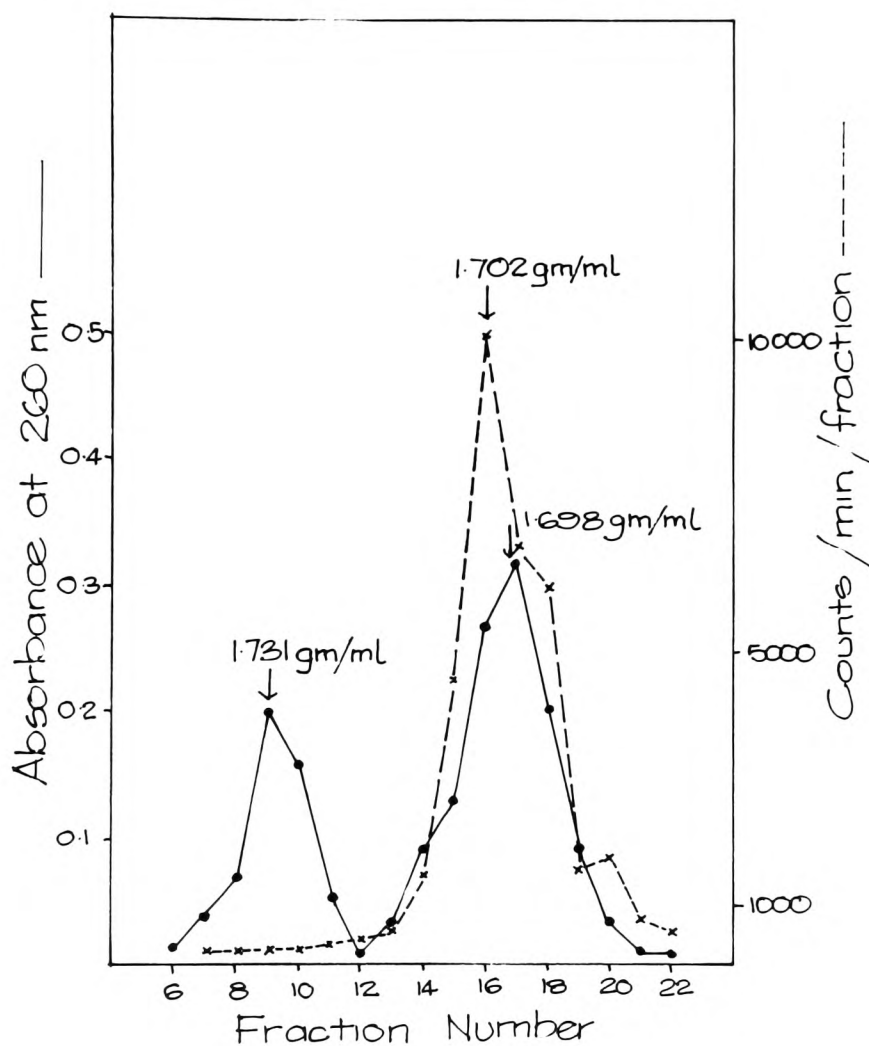


Fig. 43. Filter hybridisation result of baboon satellite cRNA to baboon DNA fractionated in CsCl. 2×10^6 counts/min of satellite cRNA was added to 0.7 ml of incubation mix and hybridisation took place at 65°C overnight.

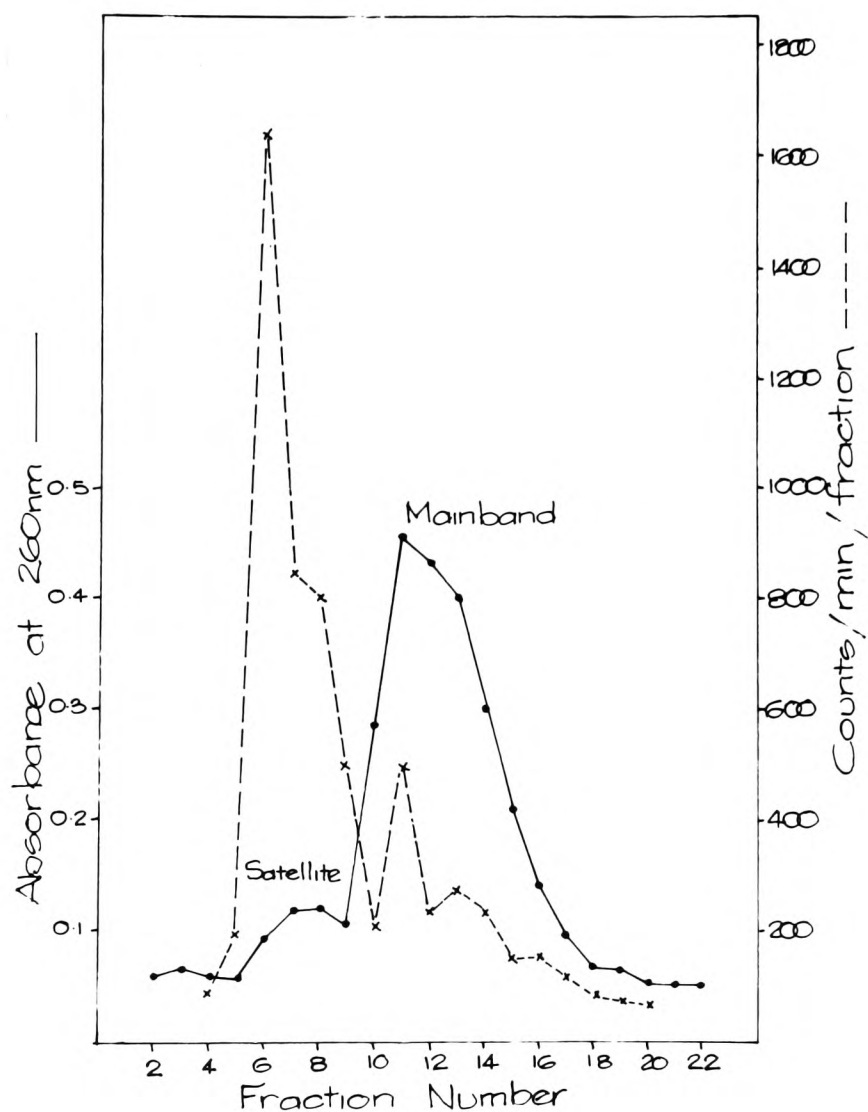


Fig. 44. Filter hybridisation result of baboon satellite cRNA to baboon DNA fractionated in $\text{Ag}^+ - \text{Cs}_2\text{SO}_4$, $R_F = 0.20$. 1.3×10^5 counts/min of satellite cRNA was added to 0.7 ml of incubation mix and hybridisation took place at 65° overnight.

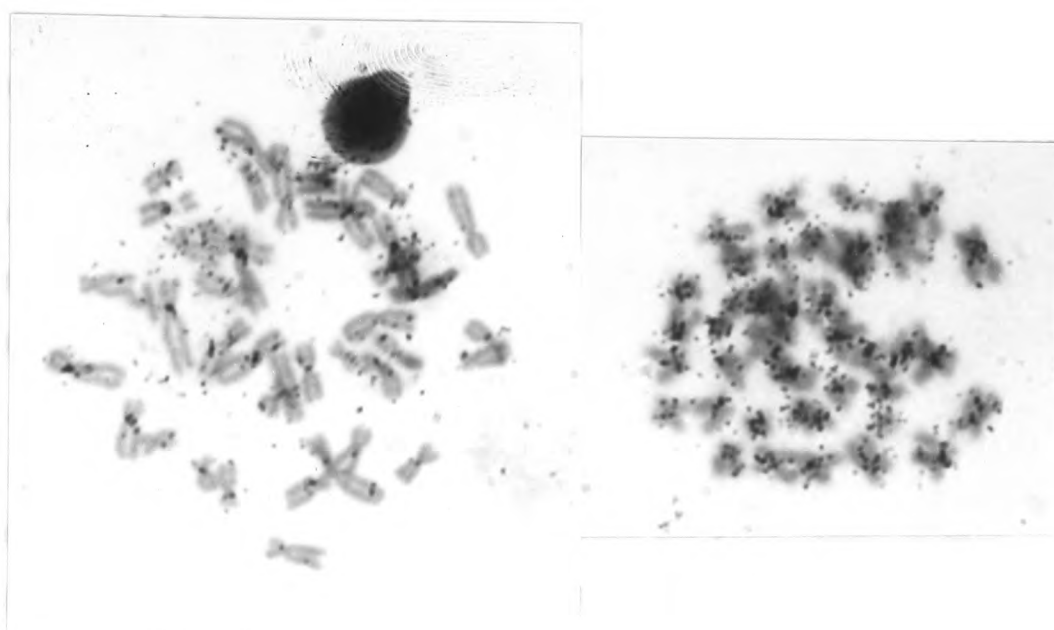


Fig. 45. In situ hybridisation result of baboon satellite cRNA hybridised to baboon metaphase chromosomes.

of reaction time. In other words at a Cot of 4.7×10^{-3} 58% renaturation had occurred indicating that baboon satellite has a low $Cot_{\frac{1}{2}}$ value, perhaps in the order of magnitude of mouse satellite which is 10^{-3} (Britten & Kohne, 1968). The satellite was 92% renatured at a Cot of 1 which suggests the presence of non-rapidly renaturing sequences in the sample. These sequences could also be inferred from the melt and remelt of the satellite. The remelt shows an increase in hyperchromicity which is 87% that of the melt indicating that at a Cot of 10^{-3} 13% of the sequences remain single stranded (or 27% if the possible effect of single strand hyperchromicity is considered). A very rapid renaturation of the whole DNA molecule does not seem to be a general property of mammalian satellite DNAs and does not occur in human, or guinea pig or in duck satellite DNAs (Corneo *et al.*, 1968a,c).

c. Transcription

12 ug of baboon satellite DNA was transcribed using all four nucleotides radioactive. Figure 42 shows the peak eluted from a Sephadex G-50 column. Fractions 13 to 16 were pooled, lyophilised and taken up in distilled water to make 0.105 ml of baboon satellite crRNA in 4 x SSC with a specific activity of 3.1×10^7 cpm/ug. The crRNA contained 2×10^3 cpm/ul.

d. Gradient hybridisation

Baboon satellite crRNA was hybridised to baboon DNA fractionated in a Cs salt density gradient. The composition of the gradient and hybridisation details are indicated in the figure legend. Baboon satellite crRNA hybridised to baboon whole DNA in CsCl to a fraction having a buoyant density of $1.702 \text{ gm/ml} \pm 0.002 \text{ gm/ml}$, which is near the native buoyant density of the satellite DNA in neutral CsCl

(Figure 43). The satellite cRNA was hybridised to baboon DNA from an $\text{Ag}^+ - \text{Cs}_2\text{SO}_4$ gradient and found to hybridise to the light side of the satellite peak (Figure 44). Since it was the extreme light side which was originally pooled to form baboon satellite free from mainband contamination, the hybridisation result suggests that this satellite too, does not consist of a homogeneous population of molecules.

e. In situ hybridisation

Baboon satellite cRNA was hybridised to baboon and mouse chromosomes. 1 μl /slide was used and hybridisation was carried out at 61°C for 8 hours. The slides were dipped in Ilford L4 liquid emulsion and stored at -4°C for 10 months. Figure 45 shows the in situ hybridisation results of baboon satellite cRNA to baboon chromosomes. Hybridisation occurred predominantly to the pericentromeric regions of most but not all chromosomes. Interphase nuclei showed grains over heterochromatic areas. It is not known why a 10 month exposure interval was necessary before localisation was apparent since the baboon satellite is relatively large, perhaps 5-10% of the genome, based on the analytical microdensitometer tracing (Figure 37).

Characterization of the baboon genome with regard to its satellite fractions is incomplete, but further work on this genome has been delayed until questions concerning human, chimpanzee and orangutan satellite DNA relationships have been researched.



Fig. 46. In situ hybridisation result of human satellite III cRNA hybridised to chimpanzee metaphase chromosomes.

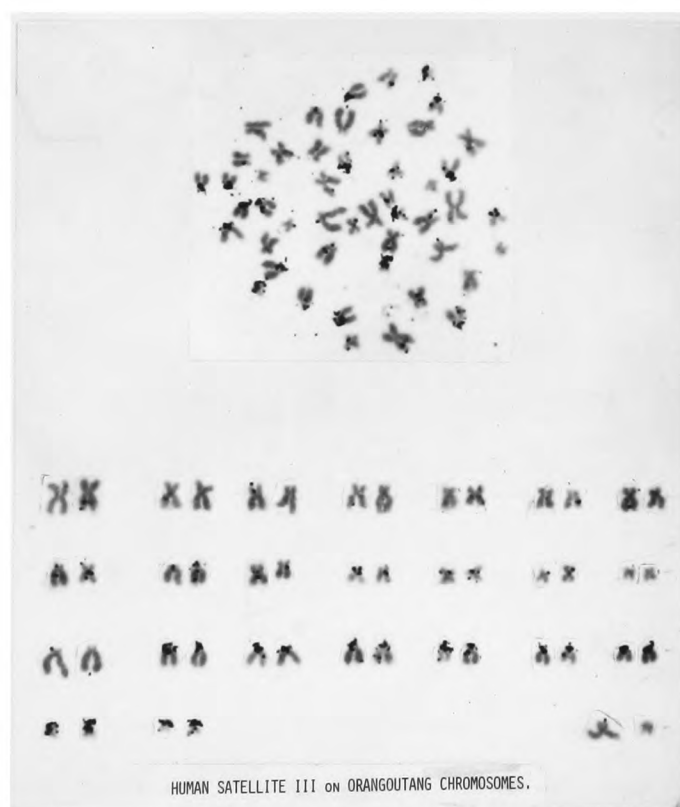


Fig. 47. In situ hybridisation result of human satellite III cRNA hybridised to orangutan metaphase chromosomes.

SECTION C. CROSS HYBRIDISATION BETWEEN SATELLITE DNAs IN FOUR PRIMATES

Introduction

The isolation of new satellite DNAs from chimpanzee and baboon genomes prompted a series of experiments to determine the relationships between the satellite sequences of the four higher primates studied. In these experiments, human satellites III and I, chimpanzee satellite A and baboon satellite cRNA were used for in situ hybridisation to metaphase chromosomes and for filter hybridisation to DNAs fractionated in Cs salt density gradients. The results of these heterologous reactions were compared with the homologous reactions reported previously in Sections A and B of the results.

Hybridisation of Human Satellite III

a. In situ

Human satellite III cRNA was hybridised to human, chimpanzee, orangutan, baboon and capuchin monkey chromosomes. As described on page 56 human satellite III was located predominantly in the centromeric heterochromatin of human chromosome 9 and to a lesser extent in the centromeric heterochromatin of the acrocentric chromosomes (Figure 8). It hybridised to the pericentromeric heterochromatin of 8 or 9 pairs of chromosomes in the chimpanzee. Figure 46 shows that hybridisation occurred mainly to one large submetacentric, one very submetacentric and three metacentric pairs, and to a lesser extent to the short arms of the acrocentric chromosomes. Human satellite III cRNA hybridised to the pericentric heterochromatin of approximately the same number of chromosomes in the orangutan (Figure 47). These results show that there are highly reiterated

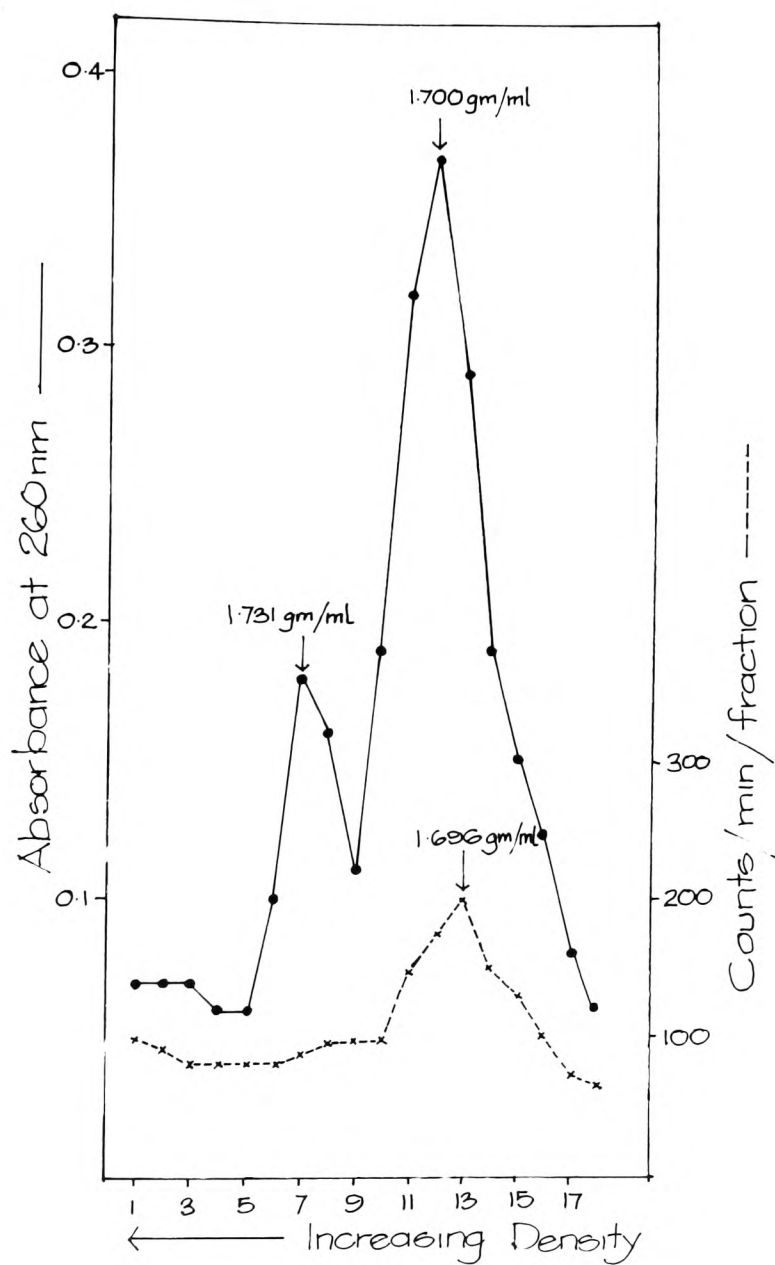


Fig. 48. Filter hybridisation result of human satellite III cRNA to chimpanzee DNA fractionated in CsCl. $1.1 - 2.1 \times 10^5$ counts/min of cRNA was added to 0.5 ml of incubation mix and hybridisation took place at 65° for 4 hours. Hybridisation was carried out by M. Moar.

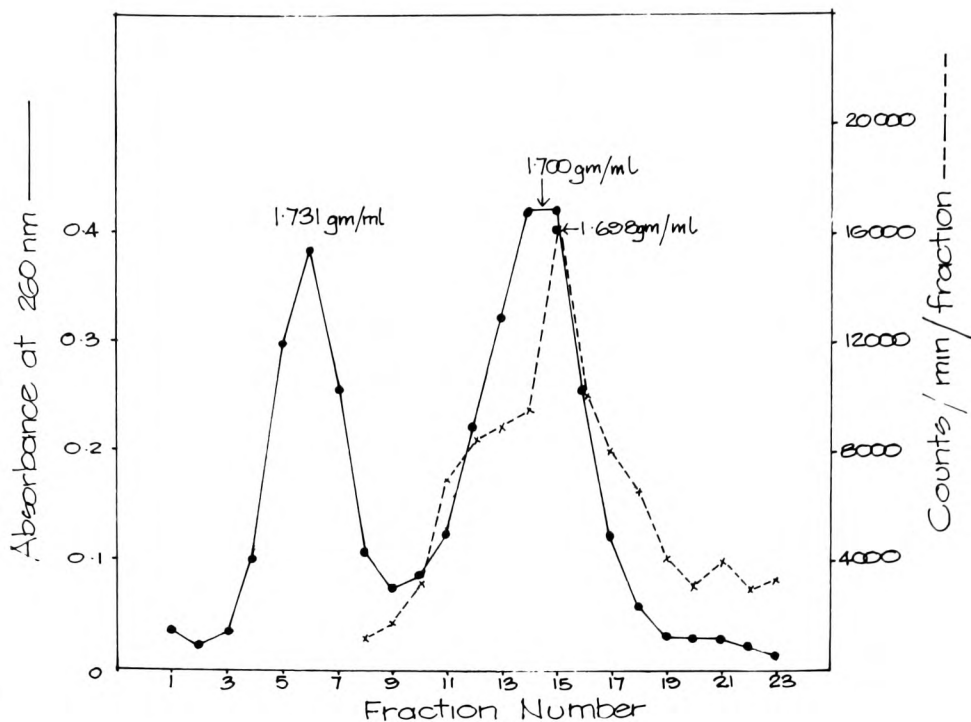


Fig. 49. Filter hybridisation result of human satellite III cRNA to orangutan DNA fractionated in CsCl. 6.6×10^5 counts/min of cRNA was added to 1 ml. incubation mix and hybridisation was carried out at 61°C for 18 hrs.

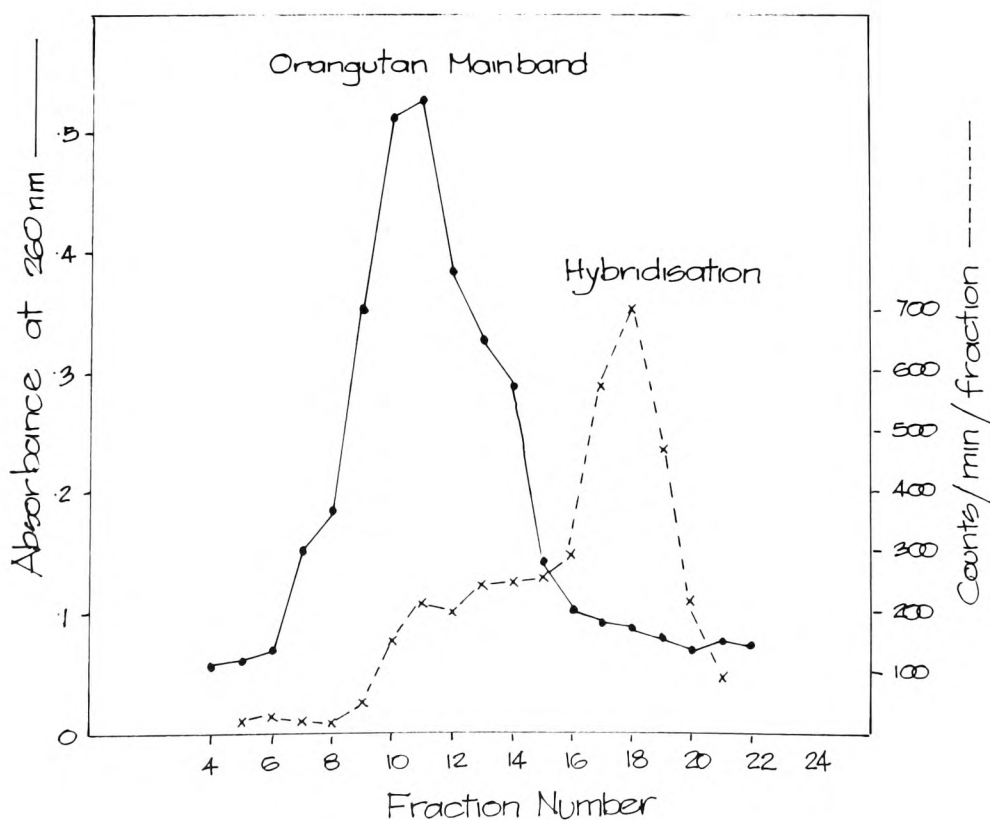


Fig. 50. Filter hybridisation of human satellite III cRNA to orangutan DNA fractionated in $\text{Ag}^+-\text{Cs}_2\text{SO}_4$, $R_F = 0.20$. 2×10^4 counts/min of cRNA was added to 0.7 ml of incubation mix and hybridisation was carried out at 65°C overnight.

sequences in pericentromeric heterochromatin in the chimpanzee and orangutan genomes which are homologous to human satellite III. Hybridisation to baboon and capuchin monkey chromosomes did not occur above background counts and the results are not illustrated.

b. Gradient hybridisation

Human satellite III cRNA was hybridised to human, chimpanzee, orangutan and baboon DNAs fractionated in Cs salt density gradients. The composition of the gradients and hybridisation details are described in the figure legends. Human satellite III cRNA hybridised to human DNA to a fraction having a buoyant density of $1.696 \text{ gm/ml} \pm .002 \text{ gm/ml}$ (Figure 6) and to human DNA from an $\text{Ag}^+ - \text{Cs}_2\text{SO}_4$ density gradient, $R_F = 0.20$, to a fraction corresponding to human satellite III DNA (Figure 7).

Human satellite III cRNA hybridised to chimpanzee DNA from a CsCl gradient to a fraction having a buoyant density of $1.696 \text{ gm/ml} \pm .002 \text{ gm/ml}$ (Figure 48) suggesting that a similar buoyant density satellite was present in this genome. The heterologous reaction was 60% less intense than the homologous reaction suggesting that changes in the related satellite sequences have occurred since the divergence of the two species.

Human satellite III cRNA hybridised to orangutan DNA from a CsCl density gradient to a fraction having a buoyant density of 1.696 gm/ml (Figure 49). $\text{Ag}^+ - \text{Cs}_2\text{SO}_4$ ($R_F = 0.25$) density gradient centrifugation of orangutan DNA produced a very broad shoulder (Figure 33) which may consist of orangutan satellite DNA obscured by low molecular weight DNA fragments. Since human satellite III hybridised to this region (Figure 50) it seems likely that an orangutan satellite DNA with a buoyant density of 1.696 gm/ml may be extracted from high molecular

weight orangutan DNA when this material is available.

Hybridisation of satellite III cRNA to baboon DNA from a CsCl gradient did not occur above background counts and is not illustrated.

Hybridisation of human satellite I

a. In situ

In situ hybridisation of human satellite I cRNA to chimpanzee and orangutan chromosomes was carried out but no hybridisation was found. The homologous hybridisation results are discussed on p. 59.

b. Gradient hybridisation

Human satellite I cRNA was hybridised to human and chimpanzee DNAs fractionated in CsCl density gradients. The composition of the gradients and the hybridisation details are described in the figure legends. Human satellite I cRNA was found to hybridise to a fraction of human DNA having a buoyant density of $1.688 \text{ gm/ml} \pm 0.002 \text{ gm/ml}$ (Figure 14) (see page 58). In this study, hybridisation did not occur to whole chimpanzee DNA or to a gradient of chimpanzee A plus B satellite DNAs in a ratio of approximately 7:3 (results not illustrated). The presence of sequences similar to human satellite I in chimpanzee DNA has not been ruled out, for they may be present in reduced numbers and/or scattered throughout the genome and thus would not be detectable by these methods. Satellite B in chimpanzee is similar to human satellite I. Their buoyant densities are low (1.683 gm/ml and 1.686 gm/ml), they constitute roughly the same proportions of the genome and they are extracted in a similar manner. They are found in association with the 1.696 gm/ml satellites of their respective species and in a similar relative position in the gradient.



Fig. 51. In situ hybridisation
result of chimpanzee satell-
ite A crNA hybridised to
human metaphase chromosomes.

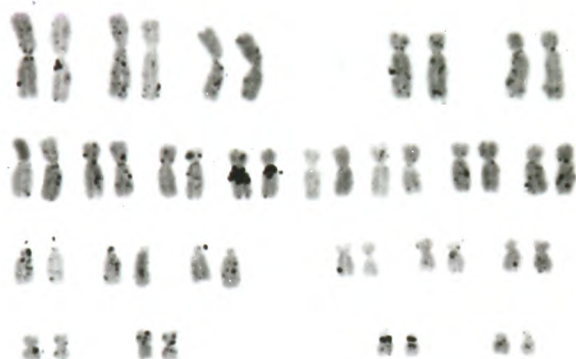


Fig. 52. In situ hybridisation
result of chimpanzee satell-
ite A crNA hybridised to
orangutan metaphase chromosomes.



CHIMPANZEE "A" SATELLITE ON ORANGOUTANG CHROMOSOMES.

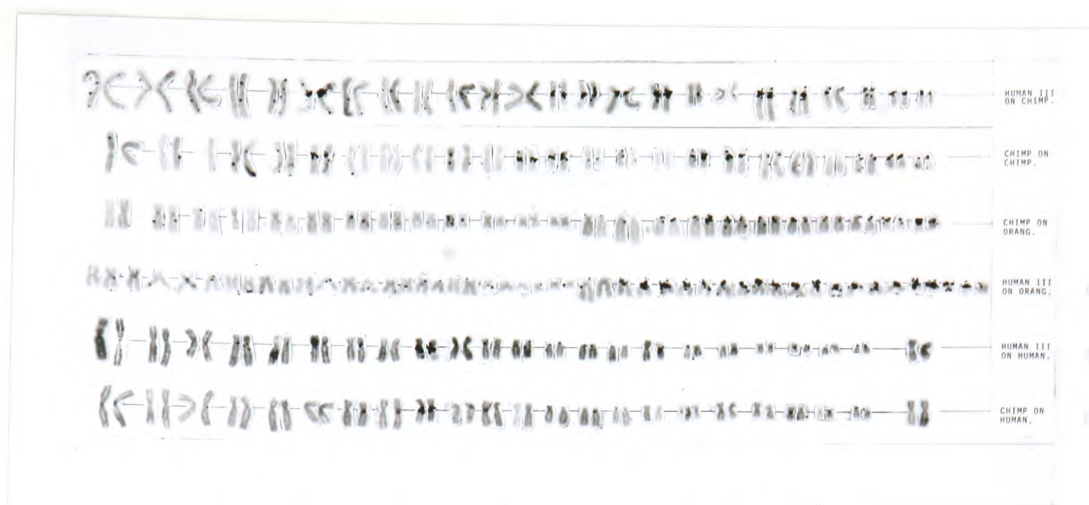


Fig. 53. Comparison of the in situ hybridisation results of human satellite III cRNA and chimpanzee satellite A cRNA to human, chimpanzee and orangutan chromosomes.

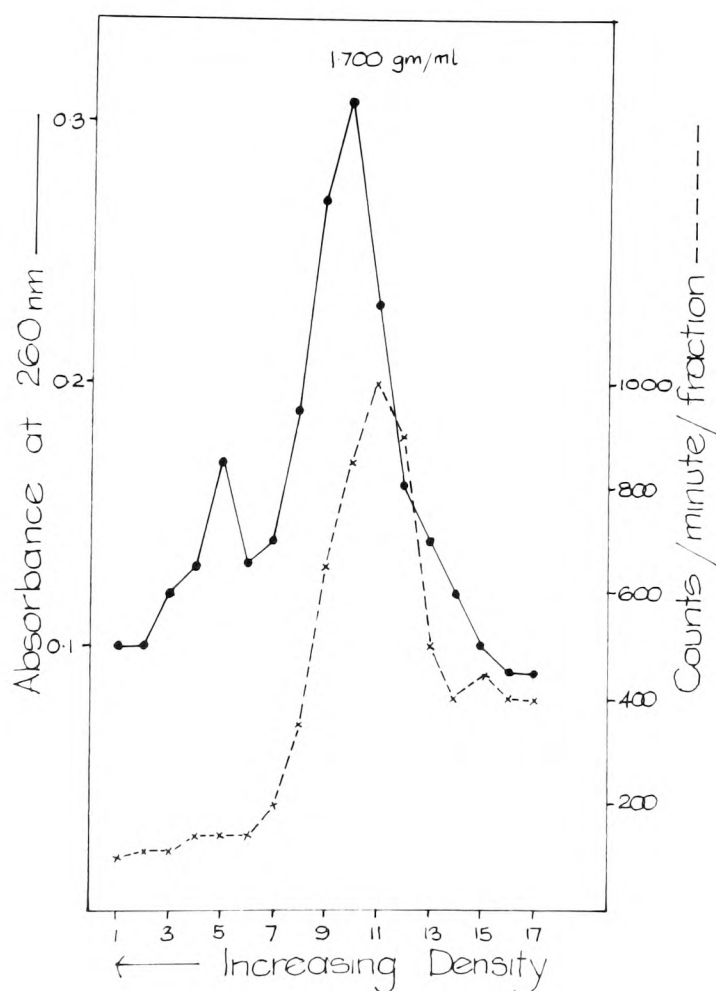


Fig. 54. Filter hybridisation of chimpanzee satellite A cRNA to human DNA fractionated in CsCl. $1.1-2.1 \times 10^5$ counts/min cRNA was added to 0.5 ml incubation mix and hybridisation was carried out at 65°C for 4 hours.

Hybridisation of chimpanzee satellite A

a. In situ

Chimpanzee satellite A cRNA was hybridised to chimpanzee, human orangutan, baboon and capuchin monkey chromosomes. Human and orangutan chromosomes showed autoradiographic patterns which were indistinguishable from those obtained using human satellite III cRNA (Figures 51 and 52). Chimpanzee chromosomes in the majority of cases examined showed a hybridisation pattern similar to that obtained with human satellite III cRNA (Figure 46), but in one or two cells with more than usually elongated chromosomes additional telomeric sites were hybridised. Figure 53 shows a comparison of the in situ hybridisation results of human satellite III and chimpanzee satellite A to human, chimpanzee and orangutan chromosomes. Hybridisation above background levels was not observed with baboon or capuchin monkey chromosomes.

b. Gradient hybridisation

Chimpanzee satellite A cRNA was hybridised to chimpanzee and human gradients fractionated in CsCl density gradients. The composition of the gradients and hybridisation details are described in the figure legends. Chimpanzee satellite A cRNA hybridised to a fraction in each of the gradients having a buoyant density of $1.696 \text{ gm/ml} \pm .002 \text{ gm/ml}$ (Figures 28 and 54). The homologous reaction is reported on page 69. The heterologous reaction was 60% less intense than the homologous which means either that there are more satellite A sequences in chimpanzee than in human, or that the human sequences have acquired changes which cause them to hybridise less efficiently.

Table 5. Comparison of the buoyant density and thermal denaturation data between human satellite III and chimpanzee satellite A.

| | Human III | | Chimpanzee A |
|-------------------------------------|-------------|-----------------------|-----------------------|
| | author | Corneo et al, 1971 | author |
| Buoyant density | | | |
| Native | 1.698 gm/ml | 1.696 gm/ml | 1.696 gm/ml |
| Denatured | 1.712 gm/ml | 1.715 gm/ml | 1.713 gm/ml |
| Renatured | 1.705 gm/ml | 1.703 gm/ml | 1.706 gm/ml |
| Separated Strands | - | 1.740 and 1.754 gm/ml | 1.755 and 1.791 gm/ml |
| T _m | | | |
| Native | 70°C | 69°C | 69°C |
| Renatured | 65°C | - | 60°C |
| ΔT_m | 5°C | 10°C* | 9°C |
| Percent increase in hyperchromicity | 35% | - | 32% |
| Native | | | |
| Renatured | 24% | - | 18% |

* Derived from Fig. 4 in Corneo et al (1971)

c. Comparison of chimpanzee satellite A and human satellite III

Table 5 summarized the buoyant density and thermal denaturation data for human satellite III and chimpanzee satellite A. The major disparity observed between the two satellites is in the ΔT_m values (the value found by the author for human satellite III is relatively low) but in other respects the two satellites are similar. In addition to the characteristics summarized in the table they resemble each other in the method of their extraction and in the fact that each is closely associated in the preparative gradient with another satellite of very low buoyant density. Cross hybridisation results of human satellite III cRNA to chimpanzee DNA and of chimpanzee satellite A cRNA to human DNA indicate that the similarity between them extends to an underlying base sequence homology. A detailed discussion of the significance of this similarity is found in Chapter 4.

Hybridisation of baboon satellite DNA

a. In situ

Baboon satellite DNA was hybridised in situ to baboon and human chromosomes. Hybridisation occurred to baboon chromosomes (described on page 76) but heterologous hybridisation was not observed after 10 months exposure.

b. Gradient hybridisation

Baboon satellite cRNA was hybridised to baboon and human DNA fractionated in CsCl density gradients. Hybridisation to baboon DNA occurred to a fraction with a buoyant density of $1.702 \text{ gm/ml} \pm 0.002 \text{ gm/ml}$ but heterologous hybridisation was not observed. It is not possible to conclude that sequences similar to baboon satellite DNA are absent from human DNA, for if only a few sequences

were present, a sufficient cot value may not have been achieved for hybridisation to occur.

The significance of the cross hybridisation results reported in this section are discussed in the following chapter.

DISCUSSION

Evolution of satellite DNA

This work describes the isolation of satellite sequences from four higher primates, including man, and reports the existence of a similar satellite in man and chimpanzee, and its probable existence in the orangutan. Human satellite III and chimpanzee satellite A have similar properties and have been shown to cross hybridise to identical positions on orangutan chromosomes and to a fraction in orangutan DNA having the same buoyant density, indicating the existence of such sequences in that species. The cross hybridisation results indicate either that the satellite was present in a common progenitor of all three species or that the satellites in the three species were independently saltated from related sequences of a family of DNA present in a common progenitor.

The great similarity between the satellites suggests either that the satellites have not had sufficient time to diverge or that they play a vital role in the genome of these higher primates. If the satellite was present in a common progenitor the minimum age of the DNA can be taken as the time since man and the three great apes diverged from a shared ancestor. Paleontological evidence estimates this time to be 15-30 million years ago (Simons, 1964; Kohne, 1970) although a later time has been suggested (Wilson and Sarich, 1969) on the basis of protein sequencing data (see Kohne, 1970, for evaluation of protein sequencing data in estimating time of divergence). If we use the paleontological evidence the minimum time during which the satellites can have diverged is 15-30 million years. There is little evidence as to whether this is long enough for sufficient nucleotide change

to have accumulated in the satellite DNA such that cross hybridisation would not be detectable between the species. Mouse and rat which diverged approximately 5 million years ago (Simpson, 1959; Sutton and McCallum, 1973) do not share a common satellite DNA. If generation time is considered instead of absolute time (and there is evidence to suggest that this is more accurate) (Rice, 1972), and if it is assumed that rodents have a generation time 1/10 that of the primates, then the equivalent divergence time for the rodent satellite would be in the order of 50 million years. Based on these assumptions it may be that the primate satellite is not sufficiently old for divergence to have occurred.

Using certain assumptions, as discussed below, it is possible to obtain an estimate of the age of a reiterated nucleotide sequence by measuring the extent to which mutation has altered the basic repeating unit. The amount of change due to the accumulation of mutations can be measured by the formation of DNA-DNA duplexes or DNA-RNA hybrids. To calculate the rate of change it is necessary to determine the number of nucleotide changes that have occurred during a known time interval. These changes can be calculated from the difference in thermal stability between native and reassociated DNA molecules: if there is no mispairing in the reassociated helix its thermal stability will approach that of native DNA (as in reassociated E.coli DNA, Britten and Kohne, 1968), but double stranded DNA containing some non-complementary base pairs will have a thermal stability lower than that of native DNA. Laird et al (1969) estimated this depression to be about 1°C per 1.5% mismatched base pairs but more recently McCarthy and Farquar (1972) estimated that the effect on thermal stability was

between 0.7° and 3.2°C for each 1% mismatched bases or 1.6°C per 1% altered bases. Using 15 million years as the time of divergence of man and the great apes and the McCarthy and Farquar figure relating ΔT_m to number of altered nucleotides, the rate of divergence of chimpanzee satellite A is found to be $0.37\%/10^6$ years and of human satellite III to be $0.21 - 0.42\%/10^6$ years depending on which estimate of the ΔT_m is used (see page 60a). These would be maximum rates of change since the satellite may have originated earlier than the time of divergence of the primates concerned, and are 2-4 times greater than the rate of $0.1 - 0.2\%/10^6$ years calculated for primate repetitive DNA (Gummerson, 1972) or $0.1\%/10^6$ years for primate non-repetitive DNA (Kohne, 1970) or $0.13\%/10^6$ years for bovine DNA (Laird et al, 1969). The rate of change of ribosomal DNA between man and chimpanzee has been calculated to be $0.04\%/10^6$ years (Jones, unpublished), a rate substantially slower than that calculated for sequences not known to be conserved.

Kohne indicates that repetitive DNA changes at the same rate as non-repetitive DNA in the animals he has studied. While this appears to be true in the mouse (Kohne, 1970) the primate satellite DNAs discussed in the present work have either changed at 2-4 times the rate of other primate DNA or they were present in an ancestor long before divergence of the species occurred. An examination of the satellite sequences in the gibbon may help to resolve this point as it diverged from a common ancestor before the divergence of man and the great apes (Simon, 1964). Yet a faster rate of change may not be unexpected for DNA, which, like satellite, is not transcribed or is transcribed and not translated. Examples of this are the non-transcribed

spacer regions between the 45s transcriptional units on rDNA of Xenopus which differ between Xenopus laevis and Xenopus mulleri while the ribosomal genes themselves are nearly identical (Brown et al, 1972) and the giant nuclear RNA of mammalian cells where the rate of accumulation of base substitutions is much greater for those parts of the precursor which are not transported to the cytoplasm (McCarthy and Farquar, 1972).

Assuming the maximum rate of mutation for chimpanzee satellite A/human satellite III as calculated above is true for all human satellites, then human satellite I would be $8-15 \times 10^6$ years old and human satellite II $3-10 \times 10^6$ years old. This would suggest (with reference to paleontological time) that human satellites I and II arose in the line leading to man, although satellite I may have arisen at the time of divergence of man from the other great apes. For this reason it will be interesting to determine if human I and chimpanzee A are sequence-related satellite DNAs. Whatever the rates of change for the primate satellite DNAs are, the ΔT_m s suggest a relative order of divergence which is consistent with the in situ hybridisation results so far achieved, that is that human III/chimpanzee A is found in man, chimpanzee and orangutan (and gorilla, Jones, unpublished) and that to date human I and II appear to be restricted to the human karyotype. A ΔT_m of 5° for baboon satellite suggests its origin to have been after baboon diverged from the lines leading to man and the great apes and, consistent with this, the satellite sequences were not found in the human genome. Estimating the age of a particular DNA assuming a constant rate of change of nucleotides even when optimal conditions of renaturation have been used (see Birnstiel,

et al., 1972) does not make allowances for DNA which may have changed at a rate substantially different from the rest of the genome. Evidence that this occurs is provided by the ribosomal cistrons which are conserved across a wide range of organisms (Brown et al., 1967) while ribosomal spacer DNA and many satellite DNA fractions appear to be species specific (Brown et al., 1972; Flamm et al., 1969b) and therefore changing at a more rapid rate. The primate satellite DNAs appear to have changed more rapidly than the rest of the primate DNA, but it is not possible to be specific since the time of saltation of the sequences is uncertain.

There remains the possibility that human III and chimpanzee A may have been independently saltated from related sequences of a family of DNA present in an early ancestor. Evidence concerning satellite DNAs from several species of the genus his has been interpreted in such a manner (Sutton and McCallum, 1973). In order to answer this question it will be necessary to separate the strands of human satellite III and chimpanzee satellite A and hybridise light human to heavy chimpanzee and the reverse. If the ΔT_{ms} of these heterologous hybrids are greater than the ΔT_{ms} of the homologous hybrids it would suggest the original sequences of both satellites were present before the time of saltation.

This may be explained in the following way. If the related satellite DNAs from several species had been present as a saltated DNA in a common progenitor, and if it is assumed that after divergence of the species the satellite DNAs in each accumulated random mutations at the same rate, then the heterologous ΔT_{ms} would be the same as the homologous ΔT_{ms} . However, if the heterologous ΔT_{ms} were found to

be greater then it suggests that the original satellite sequences existed in a common progenitor and accumulated mutations prior to their saltation.

The satellite DNAs so far located in the human genome have been situated on chromosomes A1, C9 and E16, to a lesser extent on the acrocentric chromosomes, and in the case of satellite I on the Y chromosome. It is interesting that the major locations have been the four major blocks of heterochromatin in the human genome, although the significance of this is not known. Another intriguing finding is that the three human satellites tend to share the same chromosomal locations. Various theories can be advanced to account for this. It may be that only certain "permissive" areas of the genome will accept this type of highly reiterated and perhaps intrinsically heterochromatic DNA. In this case the human satellites could be thought of as having occupied the maximum number of permissive sites and consequently be found in an association which is irrespective of their origin. If satellite DNAs can be shown to have some clear role in the nucleus then more elaborate reasons for their location may be postulated. Finally, it may be that the satellites have an evolutionary relationship to each other, that is that they have arisen one from the other, and have been translocated together to their present sites in the genome.

Britten and Kohne (1968) proposed that satellite DNA originated in a single saltatory replication and was subsequently translocated to other parts of the genome. The product of Britten's single saltation would then accumulate random mutations with the passage of time. However, Southern (1970) found that mutations in a satellite

DNA did not occur in a random manner and proposed a stepwise replication of the satellite DNA such/each step would be followed by sufficient time for random mutations to occur. At some time in this sequence of events it is possible that sufficient mutations would have occurred in the stretch of DNA selected for further replication that a new satellite DNA would be produced, bearing a certain base sequence homology to the previously replicated satellite sequences. Homology between the satellite DNAs of a single species has been demonstrated by sequencing data in Dros. virilis (Gall, in press) although it is interesting that molecular hybridisation failed to show the homology (Blumenfeld et al., 1973). How much base sequence similarity exists between the satellite DNAs in man or between the satellites of man and the other primates is not known beyond the present data.

Chromosome morphology and satellite DNA

In man Giemsa staining reveals that there are large areas of heterochromatin near the centromeres of chromosomes A1, C9, E16 and on the distal arm of the Y chromosome (Arrighi and Hsu, 1971; Chen and Ruddle, 1971; Gagne et al., 1971). The in situ hybridisation of various human satellite DNAs has been mainly to the heterochromatin of these chromosomes. Human satellite II was found chiefly in the pericentromeric heterochromatin of A1 and E16 and to a lesser extent C9 (Jones and Corneo, 1971). Human satellite III was found chiefly in C9 (Figure 8) and human satellite I was found on the distal arm of the Y chromosome and on a C group chromosome, probably chromosome 9 (Figure 16).

The location of human satellite DNAs in the heterochromatin of these chromosomes suggests a connection between specific DNAs and

differential staining in these regions. This is further illustrated by the fact that human chromosomes stained with Giemsa at pH 11 showed a distinctive differential colouration of the satellite III-rich heterochromatin of chromosome 9 in metaphase and interphase (Gagne et al, 1971; Gagne and Laberge, 1972; Bobrow et al, 1972).

Chimpanzee chromosomes (Bobrow and Madan, 1973) and orangutan chromosomes (Bobrow, personal communication to K.W. Jones) were also differentially stained in those regions which as shown here contain the related satellite sequences.

The large heterochromatic regions near the centromeres of A1, C9, E16 and on the distal arm of the Y chromosome exhibit heritable variability in size and are frequently heteromorphic in an individual (Lubs and Ruddle, 1971; Craig-Holmes and Shaw, 1971; Evans et al, 1971). The variability is sufficiently common in the population to be classified a chromosome polymorphism (Craig-Holmes et al, 1973; Craig-Holmes and Shaw, 1971; Evans et al, 1971). The frequency of the particular polymorphisms vary significantly with race (Lubs and Ruddle, 1971), however these minor variations in the amount of the heterochromatin do not appear to affect the phenotype in any discernible way (Lubs and Ruddle, 1971; Craig-Holmes and Shaw, 1971). Therefore whatever the function of the DNA located in these regions, variations in the amount present in genome have no visible phenotypic effect. If this DNA provides material for the evolution of new genes (Britten and Davidson, 1969) it is possible that individuals containing less repetitive DNA at these loci may be at an evolutionary disadvantage, although there is no evidence to support such a view.

A positive correlation between centromeric heterochromatin and rapidly renaturing DNA has been found in a variety of organisms (pages 16 to 19) but it is not known what the relationship means. In the early days of research on satellite DNAs it was suggested that centromeric heterochromatin and therefore, by association, centromeric satellite DNA conferred an advantage on chromosomes carrying it and played a part in speciation. There is a phenomenon in mice called "affinity" (Michie, 1953; Wallace, 1953) in which the centromeres of common origin in the animal are attracted to each other or to some polar element so that in meiosis the centromeres from one parent tend to go to one pole and the centromeres from the other parent tend to go to the other. In Drosophila Sandler and Novitski (1957) demonstrated the ability of "strong" centromeres to pull "weak" ones to their pole during anaphase and Lindsley and Novitski (1958) concluded it was the amount of heterochromatin adjacent to the centromeres and not the centromere itself which conferred the strength. However, the location of satellite DNAs in man and other higher primates on only a few rather than all the chromosomes in the complement, and the presence of similar satellite DNAs in related primates (pages 77 to 81) suggests that speciation and/or preferential incorporation into the egg nucleus of heterochromatin-carrying chromosomes are not functions of satellite DNA in the higher primates.

Giemsa and fluorescent banding have made possible the comparison of the primate karyotypes (Bobrow et al, 1971; Bobrow and Madan, 1973; Turleau et al, 1973). In situ hybridisation of primate satellite DNAs has explained the differences observed in several of the chromosomes selected as homologous in the higher primates. Although satellite III

hybridises to the pericentromeric heterochromatin principally of chromosome 9 in man, it hybridises to the centromeric regions of several pairs in the orangutan and chimpanzee, including in the chimpanzee a large submetacentric chromosome which is probably homologous to human 9 (Bobrow and Madan, 1973), although it lacks the large block of heterochromatin (Bobrow and Madan, 1973; Turleau et al, 1973). In the orangutan it is difficult to identify a homolog to human 9 although on the basis of Giemsa banding patterns Turleau et al (1973) selected an acrocentric chromosome lacking any large block of heterochromatin. Thus the majority of satellite III sequences in man are dispersed in the karyotypes of chimpanzee and orangutan, and at the same time the large blocks of heterochromatin on chromosome 9 is absent. The satellite III sequences common to man, chimpanzee and orangutan were originally either located on one pair of chromosomes in a common primate ancestor and subsequently spread to other chromosome pairs in chimpanzee and orangutan or they were located on several pairs of chromosomes in the common ancestor and were subsequently lost in the human genome from all but chromosome 9.

By the technique of in situ hybridisation satellite II appears to be absent from the karyotypes of the orangutan and chimpanzee. So, too, are the major blocks of heterochromatin on human chromosomes A1 and E16 in which the satellite is chiefly situated. This suggests that the acquisition of satellite II in man is associated with the appearance of two major blocks of heterochromatin. Furthermore, the age of satellite II relative to the age of satellite III (page 86) indicates that it probably arose after the divergence of man from an ancestor common to all the primates and therefore is consistent with

its restriction to the human genome.

Human satellite I also appears to be absent from the karyotypes of the chimpanzee and orangutan. This satellite, of an age intermediate between that of III and II may have arisen in the line leading to man or may have appeared at the time of divergence of the species. Satellite I is found in the large block of heterochromatin on the distal arm of the Y chromosome in man. Since this heterochromatin is absent from the karyotypes of the chimpanzee and orangutan they are probably also deficient in the satellite of this region. However satellite I is also found in the heterochromatic region of a C group chromosome, probably chromosome 9 in humans. If the satellite arose at the time of divergence of the species satellite I-like sequences may be found in the genomes of the higher primates. It remains to be established whether human I and chimpanzee A are related to each other.

List of abbreviations

| | |
|--------------------|---|
| DNA | deoxyribonucleic acid |
| RNA | ribonucleic acid |
| DNase | deoxyribonuclease |
| RNase | ribonuclease |
| DNA-P | DNA phosphate, calculated using a molecular weight of DNA of 330 daltons. Each mole of DNA would then contain 1 mole of phosphate |
| cRNA | complementary RNA, that is, RNA made <u>in vitro</u> on a DNA template |
| mRNA | messenger RNA |
| rRNA | ribosomal RNA |
| rDNA | ribosomal DNA |
| r cistron | ribosomal cistron |
| A, C, G, T, U | the bases adenine, cytosine, guanine, thymine, uracil, or their corresponding nucleotides |
| ATP, CTP, GTP, UTP | adenosine triphosphate, cytidine triphosphate, guanosine triphosphate, uridine triphosphate |
| E. coli | Escherichia coli |
| M. lys. | Micrococcus lysodeikticus |
| EDTA | ethyldiaminetetracetic acid |
| HAP | hydroxyapatite |
| SLS | sodium lauryl sulphate, a detergent |
| SSC | standard saline citrate (0.15 M NaCl and 0.015 M tri-sodium citrate pH7.1) |
| saline EDTA | 0.15 M NaCl, 0.10 M EDTA, pH8.0 |
| Tris | Tris (hydroxymethyl) aminomethane |
| TCA | trichloroacetic acid |
| PB | phosphate buffer, equimolar amounts of NaH_2PO_4 , Na_2HPO_4 |

Abbreviations (contd.)

| | |
|---------|--|
| m mole | millimole |
| gm | gram |
| mg | milligram |
| ml | milliliter |
| ug | microgram |
| μ l | microliter |
| nm | nanometer ($m\mu$, millimicron) a unit of wavelength |
| OD | optical density, normally read at 260nm |
| rpm | revolutions per minute |
| cpm | isotopic counts per minute |
| Cot | product of concentration of DNA in moles per liter x time in seconds |
| S | refers to one phase in the cell cycle, that of synthesis of DNA (the phases of the cell cycle are G_1 , S, G_2 , and M, referring respectively to Gap 1, Synthesis, Gap 2 and Mitosis) |
| Tm | mean melting temperature, defined as the temperature at which 50% of the DNA molecules are single stranded |
| R_F | molar ratio of Ag^+ or Hg^{++} bound to DNA-P |

ACKNOWLEDGEMENTS

I would like to thank Dr K.W. Jones for supervising this work and the following people for their assistance:

Professor Max Birnstiel

Bronwyn Cullen

John Gibbons

Isabella Malpica

Pat Baillie

Jeannette Muir

Ed Southern

Jim Speirs

Roger Sutcliffe

I would also like to thank the Muscular Dystrophy Association of Canada for financial support and the Wellcome Foundation for a travel grant to visit the laboratory of Professor G. Corneo, Milan, Italy.

REFERENCES

- ARRIGHI, F.E. and HSU, T.C. (1971). Localisation of heterochromatin in human chromosomes. *Cytogenetics*, 10: 81-86.
- ARRIGHI, F.E., MANDEL, M., BERGENDAHL, J. and HSU, T.C. (1970a). Buoyant densities of DNA of mammals. *Biochemical Genetics*, 4: 367-376.
- ARRIGHI, F.E., SAUNDERS, P.P. and SAUNDERS, G.F. (1970b). Chromosomal localisation of human DNA fractions by in situ hybridisation of complementary tritiated RNA. *J. Cell Biol.* 47: 9a.
- ARRIGHI, F.E., SAUNDERS, P.P., SAUNDERS, G.F. and HSU, T.C. (1971). Distribution of repetitious DNA in human chromosomes. *Experientia*, 27: 964-966.
- ASTEL, C.R., SUZUKI, D.T., KLETT, R.P., SMITH, M. and GOLDBERG, I.H. (1969). The intracellular location of the adenine- and thymine-rich component of deoxyribonucleate in testicular cells of the crab, Cancer productus. *Exptl. Cell Res.* 54: 3-10.
- BARR, M.L. (1965). Sex chromatin techniques. In: *Human Chromosome Methodology* (ed. J.J. Yunis) p.1-10, Academic Press, New York and London.
- BIRNSTIEL, M.L., CHIPCHASE, M. and SPEIRS, J. (1971). The ribosomal RNA cistrons. *Progress in Nucleic Acid Research and Molecular Biology*, 11: 351-389.
- BIRNSTIEL, M.L., SELLS, B.H. and PURDOM, I.F. (1972). Kinetic complexity of RNA molecules. *J. Mol. Biol.* 63: 21-39.
- BIRNSTIEL, M.L., SPEIRS, J., PURDOM, I.F., JONES, K. and LOENING, U.E. (1968). Properties and composition of the isolated ribosomal DNA satellite of Xenopus laevis. *Nature*, 219: 454-463.

- BISHOP, J.O. and PEMBERTON, R.E. (1972). Molecular hybridization between labelled 9s messenger RNA and DNA from the red blood cells of normal and anemic ducks. *FEBS Symposium* 24: 25-39.
- BLUMENFELD, M and FORREST, H.S. (1971). Is Drosophila dAT on the Y chromosome? *Proc. Nat. Acad. Sci.* 68: 3145-3149.
- BLUMENFELD, M. and FORREST, H.S. (1972). Differential under-replication of satellite DNAs during Drosophila development. *Nature New Biol.* 239: 170-172.
- BLUMENFELD, M., FOX, A.S. and FORREST, H.S. (1973). A family of three related satellite DNAs in Drosophila virilis. *Proc. Nat. Acad. Sci.* 70: 2772-2775.
- BOBROW, M. and MADAN, K. (1973). A comparison of chimpanzee and human chromosomes using the Giemsa-11 and other chromosome banding techniques. *Cytogenet. Cell Genet.* 12: 107-116.
- BOBROW, M., MADAN, K. and PEARSON, P.L. (1972). Staining of some specific regions of human chromosomes, particularly the secondary constriction of no.9. *Nature New Biol.* 238: 122-124.
- BOBROW, M., PEARSON, P.L. and COLLACOTT, H.E.A.C. (1971). Para-nucleolar position of the human Y chromosome in interphase nuclei. *Nature*, 232: 556-557.
- BOND, H.E., FLAMM, W.G., BURR, H.E. and BOND, S.B. (1967). Mouse satellite DNA. Further studies on its biological and physical characteristics and its intracellular localisation. *J. Mol. Biol.* 27: 289-302.
- BORST, P. and RUTTENBERG, G.J.C.M. (1966). Renaturation of mitochondrial DNA. *Biochim. Biophys. Acta*, 114: 645-647.

- BORST, P., HUTTENBERG, G.J.C.M. and KROON, A.M. (1967). Mitochondrial DNA. I. Preparation and properties of mitochondrial DNA from chick liver. *Biochim. Biophys. Acta*, 149: 140-155.
- BOSTOCK, C.J. and PRESCOTT, D.M. (1971). Buoyant density of DNA synthesized at different stages of the S phase of mouse L cells. *Exptl. Cell Res.* 64: 267-274.
- BOSTOCK, C.J., PRESCOTT, D.M. and HATCH, F.T. (1972). Timing of replication of the satellite and main band DNAs in cells of the kangaroo rat (Dipodomys ordii). *Exptl. Cell Res.* 74: 487-495.
- BRITTEN, R.J. (1968). Repeated sequences in human DNA. *Carnegie Institution Yearbook*, 67: 327-330.
- BRITTEN, R.J. (1969). Repeated DNA and transcription. In: *Problems in Biology: RNA in Development* (ed. E.W. Hanley) p.187-216, University of Utah Press, Salt Lake City.
- BRITTEN, R.J. and DAVIDSON, E.H. (1969). Gene regulation for higher cells: a theory. *Science*, 165: 349-357.
- BRITTEN, R.J. and KOHNE, D.E. (1967). Repeated nucleotide sequences. *Carnegie Institution Yearbook* 66: 73-88.
- BRITTEN, R.J. and KOHNE, D.E. (1968). Repeated sequences in DNA. *Science*, 161: 529-540.
- BROWN, D.D., WEBER, C.S. and SINCLAIR, J.H. (1967). Ribosomal RNA and its genes during oogenesis and development. *Carnegie Institution Yearbook* 66: 580-589.
- BROWN, D.D., WENSINK, P.C. and JORDAN, E. (1972). A comparison of the ribosomal DNAs of Xenopus laevis and Xenopus mulleri: the evolution of tandem genes. *J. Mol. Biol.* 63: 57-73.

- BROWN, J.E. and JONES, K.W. (1972). Localisation of satellite DNA in the microchromosomes of the Japanese quail by in situ hybridisation. *Chromosoma* (Berl.) 38: 313-318.
- BROWN, S.W. (1966). Heterochromatin. *Science*, 151: 417-425.
- CHEN, T.R. and RUDDLE, F.H. (1971). Karyotype analysis utilizing differentially stained constitutive heterochromatin of human and marine chromosomes. *Chromosoma* (Berl.) 34: 51-72.
- CHENG, T.Y. and SUEOKA, N. (1964). Polymer similar to polydeoxy-adenylate-thymidylate in various tissues of a marine crab. *Science*, 143: 1442-1443.
- CHERVENKA, C.H. (1969). A Manual of Methods for the Analytical Ultracentrifuge. Published by Spinco Division of Beckman Instruments, Palo Alto, California.
- CHUN, E.H. and LITTLEFIELD, J.W. (1963). The replication of the minor DNA component of mouse fibroblasts. *J. Mol. Biol.* 7: 245-248.
- CHUN, E.H., VAUGHAN, M.H. and REICH, A. (1963). The isolation and characterisation of DNA associated with chloroplast preparations. *J. Mol. Biol.* 7: 130-141.
- COHEN, A.K., HUH, T.Y. and HELLEINER, C.W. (1973). Transcription of satellite DNA in mouse L-cells. *Can. J. Biochem.* 51: 529-532.
- COMINGS, D.E. and MATTOCCIA, E. (1970a). Studies of microchromosomes and a G-C rich DNA satellite in the quail. *Chromosoma* (Berl.) 30: 202-214.
- COMINGS, D.E. and MATTOCCIA, E. (1970b). Replication of repetitious DNA and the S period. *Proc. Nat. Acad. Sci.* 67: 448-455.
- CORNEO, G., GINELLI, E. and POLLI, E. (1967). A satellite DNA isolated from human tissues. *J. Mol. Biol.* 23: 619-622.

- CORNEO, G., GINELLI, E. and POLLI, E. (1968a). Isolation of the complementary strands of a human satellite DNA. *J. Mol. Biol.* 33: 331-335.
- CORNEO, G., GINELLI, E. and POLLI, E. (1968b). Presence of a satellite DNA in normal and leukemic human tissues. *Acta haemat.* 39: 75-84.
- CORNEO, G., GINELLI, E. and POLLI, E. (1970a). Repeated sequences in human DNA. *J. Mol. Biol.* 48: 319-327.
- CORNEO, G., GINELLI, E. and POLLI, E. (1970b). Different satellite deoxyribonucleic acids of guinea pig and ox. *Biochemistry*, 9: 1565-1571.
- CORNEO, G., GINELLI, E. and POLLI, E. (1971). Renaturation properties and localisation in heterochromatin of human satellite DNAs. *Biochim. Biophys. Acta* 247: 528-534.
- CORNEO, G., MOORE, C., SANADI, D.R., GROSSMAN, L.J. and MARMUR, J. (1966). Mitochondrial DNA in yeast and some mammalian species. *Science*, 151: 687-689.
- CORNEO, G., GINELLI, E., SOAVE, C. and BERNARDI, G. (1968c). Isolation and characterization of mouse and guinea pig satellite DNAs. *Biochemistry*, 70: 4373-4379.
- CORNEO, G., ZARDI, L. and POLLI, E. (1972). Elution of human satellite DNAs on a methylated albumin kieselguhr chromatographic column: isolation of satellite DNA IV. *Biochim. Biophys. Acta*, 269: 201-204.
- COUDRAY, Y., QUETIER, F. and GUILLE, E. (1970). New compilation of satellite DNAs. *Biochim. Biophys. Acta*, 217: 259-267.
- CRAIG-HOLMES, A.P., MOORE, F.B. and SHAW, M.W. (1973). Polymorphism of human C-band heterochromatin. I. Frequency of variants. *Am. J. Hum. Gen.* 25: 181-192.

- CRAIG-HOLMES, A.P. and SHAW, M.W. (1971). Polymorphism of human constitutive heterochromatin. *Science*, 174: 702-704.
- DUERKSON, J.D. and MCCARTHY, B.J. (1971). Distribution of deoxy-ribonucleic acid sequences in fractionated chromatin. *Biochemistry*, 10: 1471-1478.
- ECKHARDT, R.A. and GALL, J.G. (1971). Satellite DNA associated with heterochromatin in Rhynchosciara. *Chromosoma (Berl.)* 32: 407-427.
- ETINGH, T.D. (1970). DNA hybridisation in the genus Drosophila. *Genetics*, 66: 55-68.
- EVANS, H.J., BUCKTON, K.E. and SUMNER, A.T. (1971). Cytological mapping of human chromosomes: results obtained with guinacrine fluorescence and the acetic-saline-giemsa technique. *Chromosoma (Berl.)* 35: 310-325.
- FANSLER, B., TRAVAGLINI, E., LOEB, L. and SCHULTZ, J. (1970). Structure of Drosophila melanogaster dAT replicated in an in vitro system. *Biochem. Biophys. Res. Commun.* 40: 1266-1272.
- FLAMM, W.G., BERNHEIM, N.J. and BRUBAKER, P.E. (1971). Density gradient analysis of newly replicated DNA from synchronised mouse lymphoma cells. *Exptl. Cell Res.* 64: 97-104.
- FLAMM, W.G., BIRNSTIEL, M.L. and WALKER, P.M.B. (1966a). Preparation and fractionation and isolation of single strands of DNA by isopycnic ultracentrifugation in fixed-angle rotors. In: *Subcellular Components: Preparation and Fractionation* (ed. G.D. Birnie and S.M. Fox) p.125-155, Butterworths, London.
- FLAMM, W.G., BOND, H.E. and BURR, H.E. Density gradient centrifugation of DNA in a fixed-angle rotor. A higher order of resolution. *Biochim. Biophys. Acta*, 129: 310-317.

FLAMM, W.G., BOND, H.E., BURR, H.E. and BOND, S.B. (1966b).

Satellite DNA isolated from mouse liver; some physical and metabolic properties. *Biochim. Biophys. Acta*, 123: 652-654.

FLAMM, W.G., McCALLUM, M. and WALKER, P.M.B. (1967). The isolation of complementary strands from a mouse DNA fraction. *Proc. Nat. Acad. Sci.* 57: 1729-1734.

FLAMM, W.G., WALKER, P.M.B. and McCALLUM, M. (1969b). Some properties of the single strands isolated from the DNA of the nuclear satellite of the mouse (Mus musculus). *J. Mol. Biol.* 40: 423-443.

GAGNE, R. and LABERGE, C. (1972). Specific cytological recognition of the heterochromatic segment of number 9 chromosome in man. *Exptl. Cell Res.* 73: 239-242.

GAGNE, R., TANGUAY, R. and LABERGE, C. (1971). Differential staining patterns of heterochromatin in man. *Nature New Biol.* 232: 29-30.

GALL, J.G. (1970). The ribosomal cistrons in Dytiscid beetles. *J. Cell Biol.* 47: 68a.

GALL, J.G. (in press). In: *Symposium on Molecular Cytogenetics*. Pergamon Press, Elmsford, N.Y. (1971). Cited in Blumenfeld et al (1973).

GALL, J.G., COHEN, E.H. and POLAN, M.L. (1971). Repetitive DNA sequences in Drosophila. *Chromosoma (Berl.)* 33: 319-344.

GALL, J.G., MacGREGOR, H.C. and KIDSTON, M.E. (1969). Gene amplification in the oocytes of Dytiscid water beetles. *Chromosoma (Berl.)* 26: 169-187.

GIBOR, A. and GRANIK, S. (1964). Plastids and mitochondria: inheritable systems. *Science*, 145: 890-897.

GIBSON, I. and HEWITT, G. (1970). Isolation of DNA from B chromosomes in grasshoppers (Myrmeleotettix maculatus). *Nature*, 225: 67-68.

- GIBSON, I. and HEWITT, G. (1972). Interpopulation variation in the satellite DNA from grasshoppers with B-chromosomes. *Chromosoma* (Berl.) 38: 121-138.
- GRAHAM, D.E. and SKINNER, D.M. (1970). Conservation of nucleotide sequences in d(G+C)-rich satellite DNAs of Crustacea. *J. Cell. Biol.* 47: 76a.
- GRAHAM, D.E. and SKINNER, D.M. (1973). Homologies of repetitive DNA sequences among Crustacea. *Chromosoma* (Berl.) 40: 135-152.
- GREENBERG, L.J. and UHR, J.W. (1967). Myeloma tumor satellite DNA: a role in ribosomal RNA synthesis. *Biochem. Biophys. Res. Commun.* 27: 523-528.
- GUMMERSON, K.S. (1972). The evolution of repeated DNA in primates. Ph.D. thesis. Johns Hopkins University, Baltimore, Maryland.
- HAREL, J., HANANIA, N., TAPIERO, H. and HAREL, L. (1968). RNA replication by nuclear satellite DNA in different mouse cells. *Biochem. Biophys. Res. Commun.* 33: 696-701.
- HATCH, F.T. and MAZIRIMAS, J.A. (1970). Satellite DNAs in the kangaroo rat. *Biochim. Biophys. Acta*, 224: 291-294.
- HATFIELD, J.M.R. and WALKER, P.M.B. (1973). Satellite DNA replication in baby mouse kidney cells infected with polyoma virus. *Nature New Biol.* 242: 141-142.
- HENNIG, W. (in press). Molecular hybridisation of DNA and RNA in situ. *International Review of Cytology*.
- HENNIG, W., HENNIG, I. and STEIN, H. (1970). Repeated sequences in the DNA of Drosophila and their localization in giant chromosomes. *Chromosoma*, (Berl.) 32: 31-63.
- HENNIG, W. and MEER, B. (1971). Reduced polyteny of ribosomal RNA cistrons in giant chromosomes of Drosophila hydei. *Nature New Biol.* 233: 70-72.

- HENNIG, W. and WALKER, P.M.B. (1970). Variations on the DNA from two rodent families (Cricetidae and Muridae). *Nature*, 225: 915-919.
- HSU, T.C., ARRIGHI, F.E. and SAUNDERS, G.F. (1972). Compositional heterogeneity of human heterochromatin. *Proc. Nat. Acad. Sci.* 69: 1464-1466.
- HUANG, C-C. (1971). Replication of euchromatin and heterochromatin in a mealy bug. *Chromosoma (Berl.)* 34: 230-242.
- INGLE, J., PEARSON, G.G. and SINCLAIR, J. (1973). Species distribution and properties of nuclear satellite DNA in higher plants. *Nature New Biol.* 242: 193-197.
- JACOB, J. (1971). The Practice and Application of Electron Microscope Autoradiography. *International Review of Cytology*, 30: 91-181.
- JENSEN, R.H. and DAVIDSON, N. (1966). Spectrophotometric, potentiometric and density gradient ultracentrifugation studies of the binding of silver ion by DNA. *Biopolymers*, 4: 17-32.
- JONES, K.W. (1970). Chromosomal and nuclear location of mouse satellite DNA in individual cells. *Nature*, 225: 912-915.
- JONES, K.W. (1973). The method of in situ hybridization. In: *New Techniques in Biophysics and Cell Biology*, Vol. 1 (ed. R.H. Pain and B.J. Smith) p.29-66, John Wiley and Sons, London.
- JONES, K.W. and CORNEO, G. (1971). Location of satellite and homogeneous DNA sequences on human chromosomes. *Nature New Biol.* 233: 268-271.
- JONES, K.W., PROSSER, J., CORNEO, G., GINELLI, E. and BOBROW, M. (1972). Satellite DNA constitutive heterochromatin and human evolution. In: *Modern Aspects of Cytogenetics: Constitutive Heterochromatin*

- in Man. Symposia Medica Hoechst 6, (ed. R.A. Pfeiffer) p. 45-74.
F.K. Schattauer Verlag, Stuttgart and New York.
- KIRBY, K.S. (1957). A new method for the isolation of deoxyribonucleic acids: Evidence on the nature of bonds between deoxyribonucleic acid and protein. Biochem. J. 66, 495-504.
- KIT, S. (1961). Equilibrium sedimentation in density gradients of DNA preparations from animal tissues. J. Mol. Biol. 3: 711-716.
- KIT, S. (1962). Species differences in animal DNAs as revealed by equilibrium sedimentation in density gradients. Nature, 193: 274-275.
- KLETT, P.R., CHABOT, I.M., SMITH, M. and HART, J.F.L. (1969). Deoxyribonucleates of crabs of the super family Brachyrrhyncha. Can. J. Biochem. 47: 829-832.
- KOHNE, D.E. (1970). Evolution of higher organism DNA. Quarterly Review of Biophysics, 33: 327-375.
- LAIRD, C.D. and MCCARTHY, B.J. (1968). Magnitude of interspecific nucleotide sequence variability in Drosophila. Genetics, 60: 303-322.
- LAIRD, C.D., McCONAUGHY, B.L. and MCCARTHY, B.J. (1969). Rate of fixation of nucleotide substitutions in evolution. Nature, 224: 149-154.
- LEVAN, A., HSU, T.C. and STICH, H.F. (1962). The idiogram of the mouse. Hereditas, 48: 677-687.
- LIMA-DE-FARIA, A., BIRNSTIEL, M. and JAWORSKA, H. (1969). Amplification of ribosomal cistrons in the heterochromatin of Acheta. Genetics, 61 (Suppl.): 145-159.

- LINDSLEY, D.L. and NOVITSKI, E. (1958). Localization of genetic factors responsible for the kinetic activity of X chromosomes of Drosophila melanogaster. *Genetics*, 43: 790-798.
- LUBS, H.A. and RUDDLE, F.H. (1971). Chromosome polymorphism in American Negro and white populations. *Nature*, 233: 134-136.
- MAIO, J.J. (1971). DNA strand reassociation and polyribonucleotide binding in the African green monkey, Cercopithecus aethiops. *J. Mol. Biol.* 56: 579-595.
- MAIO, J.J. and SCHILDKRAUT, C.L. (1967). Isolated mammalian metaphase chromosomes. I. General characteristics of nucleic acids and proteins. *J. Mol. Biol.* 24: 29-39.
- MAIO, J.J. and SCHILDKRAUT, C.L. (1969). Isolated mammalian metaphase chromosomes. II. Fractionated chromosomes of mouse and Chinese hamster cells. *J. Mol. Biol.* 40: 203-216.
- MANDEL, M. and MARMUR, J. (1968). Use of ultraviolet absorbance - temperature profile for determining guanine plus cytosine content of DNA. *Methods in Enzymology*, 12B: 195-206.
- MARMUR, J. (1961). A procedure for the isolation of deoxyribonucleic acid from micro-organisms. *J. Mol. Biol.* 3: 208-218.
- MARMUR, J. and Doty, P. (1959). Heterogeneity in deoxyribonucleic acids. I. Dependence on composition of the configurational stability of deoxyribonucleic acids. *Nature*, 183: 1427-1429.
- MARMUR, J. and DOTY, P. (1961). Thermal renaturation of deoxyribonucleic acids. *J. Mol. Biol.* 3: 585-594.
- MATTOCCIA, E. and COMINGS, D.E. (1971). The buoyant density and satellite composition of DNA of mouse heterochromatin. *Nature New Biol.* 229: 175-176.

- MAZRIMAS, J.A. and HATCH, F.T. (1970). Intranuclear distribution of satellite DNA from kangaroo rat. *Exptl. Cell Res.* 63: 462-466.
- MAZRIMAS, J.A. and HATCH, F.T. (1972). A possible relationship between satellite DNA and the evolution of the kangaroo rat species (genus Dipodomys). *Nature New Biol.* 240: 102-105.
- MCCARTHY, B.J. and FARQUAR, M.N. (1972). The rate of change of DNA in evolution. *Brookhaven Symposium*, 23: 1-43.
- McCONAUGHY, B.L. and MCCARTHY, B.J. (1970). Related base sequences in the DNA of simple and complex organisms. VI. The extent of base sequence divergence among the DNAs of various rodents. *Biochemical Genetics*, 4: 425-446.
- MacGREGOR, H.C. and KEZER, J. (1971). The chromosomal localisation of a heavy satellite DNA in the testis of Plethodon c. cinereus. *Chromosoma (Berl.)* 33: 167-182.
- McLAREN, A. and WALKER, P.M.B. (1966). Discriminating power of rodent deoxyribonucleic acid on incubation in agar. *Nature*, 211: 486-490.
- MICHIE, D. (1953). Affinity: a new genetic phenomenon in the house mouse. Evidence from distant crosses. *Nature*, 171: 26-27.
- MILLER, O.Z. and BEATTY, B.R. (1969). Extrachromosomal nucleolar genes in amphibian oocytes. *Genetics*, 61 (suppl.1): 133-143.
- MITCHELL, A. Personal Communication.
- MIYAZAWA, Y. and THOMAS, C.A. (1965). Nucleotide composition of short segments of DNA molecules. *J. Mol. Biol.* 11: 223-237.
- NANDI, U.S., WANG, J.C. and DAVIDSON, N. (1965). Separation of deoxyribonucleic acids by Hg (II) binding and Cs_2SO_4 density-gradient centrifugation. *Biochemistry*, 4: 1687-1696.
- NAPIER, J.R. and NAPIER, P.H. (1967). A Handbook of Living Primates. Academic Press, London and New York.

- NATARAJAN, A.T. and AHNSTROM, G. (1970). The localisation of radiation induced chromosome aberrations in relation to distribution heterochromatin in Secale cereale. *Chromosoma* (Berl.) 30: 250-257.
- OHTA, T. and KIMURA, M. (1971). Functional organization of genetic material as a product of molecular evolution. *Nature*, 233: 118-119.
- PARDUE, M.L. and GALL, J.G. (1970). Chromosomal localization of mouse satellite DNA. *Science*, 168: 1356-1358.
- PARDUE, M.L., GERBI, S.A., ECKHARDT, R.A. and GALL, J.G. (1970). Cytological localization of DNA complementary to ribosomal RNA in polytene chromosomes of Diptera. *Chromosoma* (Berl.) 29: 268-290.
- RICE, N.R. (1972). Change in repeated DNA in evolution. *Brookhaven Symposium*, 23: 44-79.
- SANDLER, L. and NOVITSKI, E. (1957). Meiotic drive as an evolutionary force. *Am. Naturalist*, 91: 105-110.
- SAUNDERS, G.F., HSU, T.C., GETZ, M., SIMES, E.L. and ARRIGHI, F.E. (1972a). Locations of a human satellite DNA in human chromosomes. *Nature New Biol.* 236: 244-246.
- SAUNDERS, G.F., SHIRAKAWA, S., SAUNDERS, P.P., ARRIGHI, F.E. and HSU, T.C. (1972b). Populations of repeated DNA sequences in the human genome. *J. Mol. Biol.* 63: 323-334.
- SCHILDKRAUT, C.L. and MAIO, J.J. (1968). Studies on the intra-nuclear distribution and properties of mouse satellite DNA. *Biochim. Biophys. Acta*, 161: 76-93.
- SCHILDKRAUT, C.L. and MAIO, J.J. (1969). Fractions of HeLa differing in their content of guanine + cytosine. *J. Mol. Biol.* 46: 305-312.

- SCHILDKRAUT, C.L., MARMUR, J. and DOTY, P. (1962). Determination of the base composition of deoxyribonucleic acid from its buoyant density in CsCl. *J. Mol. Biol.* 4: 430-443.
- SCHMID, W. and LEPPERT, M.F. (1969). Rates of DNA synthesis in heterochromatic and euchromatic segments of the chromosome complements of two rodents. *Cytogenetics*, 8: 125-135.
- SHERMAN, M.I., McLAREN, A. and WALKER, P.M.B. (1972). Mechanism of accumulation of DNA in giant cells of mouse fibroblast. *Nature New Biol.* 238: 175-176.
- SIMONS, E.L. (1964). The early relatives of man. *Scient. Am.* 211: 50-62.
- SIMPSON, G.G. (1959). The nature and origin of supraspecific taxa. *Cold Spring Harbor Symp. Quant. Biol.* 24: 255-271.
- SKINNER, D.M. (1967). Satellite DNAs in the crabs Gecarcinus lateralis and Cancer pagurus. *Proc. Nat. Acad. Sci.* 58: 103-110.
- SKINNER, D.M., BEATTIE, W.G., KERR, M.S. and GRAHAM, D.E. (1970). Satellite DNAs in Crustacea: Two different components with the same density in neutral CsCl gradients. *Nature*, 227: 837-839.
- SKINNER, D.M. and TRIPLETT, L.L. (1967). The selective loss of DNA satellites on deproteinization with phenol. *Biochem. Biophys. Res. Commun.* 28: 892-897.
- SMITH, B.J. (1970). Light satellite-band DNA in mouse cells infected with polyoma virus. *J. Mol. Biol.* 47: 101-106.
- SMITH, M. (1963). Deoxyribonucleic acids in crabs of the genus Cancer. *Biochem. Biophys. Res. Commun.* 10: 67-72.
- SMITH, M. (1964). Deoxyribonucleic acids of Crustacea. *J. Mol. Biol.* 9: 17-23.

- SOUTHERN, E.M. (1970). Base sequence and evolution of guinea pig α -satellite DNA. *Nature*, 227: 794-798.
- SPEIRS, J. (1972). Biophysical characterization of ribosomal DNA of Xenopus laevis. Ph.D. thesis, Edinburgh University.
- STAFFORD, D.R. and GUILD, W.R. (1969). Satellite DNA from sea urchin sperm. *Exptl. Cell Res.* 55: 347-350.
- STEFFENSEN, D.M. and WIMBER, D.E. (1972). Hybridization of nucleic acids to chromosomes. In: *Nucleic Acid Hybridisation in the Study of Cell Differentiation*. (Ed. H. Ursprung) p.47-63 Springer-Verlag, Berlin and New York.
- SUEOKA, N., MARMUR, J. and DOTY, P. (1959). II. Dependence of the density of deoxyribonucleic acids on guanine-cytosine content. *Nature*, 183: 1429-1431.
- SUEOKA, N. (1961). Variation and heterogeneity of base composition of DNA: a compilation of old and new data. *J. Mol. Biol.* 3: 31-40.
- SUEOKA, N. and CHENG, T. (1962). Natural occurrence of a deoxyribonucleic acid resembling the deoxyadenylate-deoxythymidylate polymer. *Proc. Nat. Acad. Sci.* 48: 1851-1856.
- SUTTON, W.D. and McCALLUM, M. (1972). Related satellite DNAs in the genus Mus. *J. Mol. Biol.* 71: 633-656.
- SWARTZ, M.N., TRAUTNER, J.A. and KORNBERG, A. (1962). Enzymatic synthesis of deoxyribonucleic acid. XI. Further studies on nearest neighbour base sequences in deoxyribonucleic acids. *J. Biol. Chem.* 237: 1961-1967.
- TOBIA, A., SCHILDKRAUT, C.L. and MAIO, J.J. (1970). Deoxyribonucleic acid replication in synchronized cultured mammalian cells. I. Time of synthesis of molecules of different average guanine + cytosine content. *J. Mol. Biol.* 54: 499-515.

- TURLEAU, C., deGROUCHY, J. and KLEIN, M. (1973). Phylogenie chromosomique de l'homme et des primates hominiens. Cahiers de Medecine, 14: 367-379.
- VINOGRAD, J.J., MORRIS, N., DAVIDSON, N. and DOVE, W.F. (1963). The buoyant behaviour of viral and bacterial DNA in alkaline CsCl. Proc. Nat. Acad. Sci. 49: 12-17.
- WALKER, P.M.B. (1968). How different are the DNAs from related animals? Nature, 219: 228-232.
- WALKER, P.M.B. (1969). The specificity of molecular hybridization in relation to studies on higher organisms. Prog. in Nucleic Acid Res. and Molecular Biol. 9: 301-326.
- WALKER, P.M.B. (1971). "Repetitive" DNA in higher organisms. Progress in Biophysics and Molecular Biology, 23: 145-190.
- WALKER, P.M.B., FLAMM, W.G. and McLAREN, A. (1969). Highly repetitive DNA in rodents. In: Handbook of Molecular Cytology. (Ed. A.Lima-de-Faria) p.53-66. North Holland Publishing Company, Amsterdam and London).
- WALLACE, M.E. (1953). Affinity: a new genetic phenomenon in the house mouse. Evidence from within laboratory stocks. Nature, 171: 27-28.
- WARING, M. and BRITTEN, R.J. (1966). Nucleotide sequence repetition: a rapidly reassociating fraction of mouse DNA. Science, 154: 791-794.
- WATSON, J.D. (1965). Molecular Biology of the Gene. W.A. Benjamin Inc., New York, and Amsterdam.
- WILSON, A.C. and SARICH, V.M. (1969). A molecular time scale for human evolution. Proc. Nat. Acad. Sci. 63: 1088-1093.
- YASMINEH, W.G. and YUNIS, J.J. (1969). Satellite DNA in mouse autosomal heterochromatin. Biochem. Biophys. Res. Commun. 35: 779-782.

- YASMINEH, W.G. and YUNIS, J.J. (1970). Localization of mouse satellite DNA in constitutive heterochromatin. *Exptl. Cell Res.* 59: 69-75.
- YASMINEH, W.G. and YUNIS, J.J. (1971). Satellite DNA in calf heterochromatin. *Exptl. Cell Res.* 64: 41-48.
- YUNIS, J.J. and YASMINEH, W.G. (1970). Satellite DNA in constitutive heterochromatin in the guinea pig. *Science*, 168: 263-265.
- PROSSER, J., MOAR, M., BOBROW, M. and JONES, K.W. (1973). Satellite sequences in chimpanzee (Pan troglodytes). *Biochim. Biophys. Acta*, 319: 122-134.
- VINOGRAD, J. and HEARST, J.E. (1962). Equilibrium sedimentation of macromolecules and viruses in a density gradient. *Progress in the Chemistry of Organic Natural Products*, 20: 372-421.

Reprinted from

Biochimica et Biophysica Acta, 000 (1973) 122-134

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BBA 97764

SATELLITE SEQUENCES IN CHIMPANZEE (*PAN TROGLODYTES*)

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(Received February 28th, 1973)

SUMMARY

Chimpanzee DNA was found to contain two satellite fractions when centrifuged in Ag^+ - Cs_2SO_4 density gradients: satellite A with a buoyant density of 1.696 gm/cm^3 and satellite B with a buoyant density of 1.683 gm/cm^3 . It was shown that satellite A shares many of the physical properties of human satellite III, and cross hybridisation results indicate the presence of an underlying base sequence homology. The original satellite sequence was presumably present in a common progenitor of both these primates and is therefore of interest to human evolution. This is the first report of cross hybridisation between two such distantly related species, demonstrating an interfamilial hybridisation of satellite DNA among higher primates.

INTRODUCTION

The DNA of many eukaryotes is known to contain repeated sequences^{1,2}. These repeated sequences are sometimes separated from the bulk of the DNA by isopycnic centrifugation in caesium salt gradients and as such are called satellite DNA fractions^{3,4}. The present work describes the characterization of two satellite fractions from chimpanzee DNA, one of which shows homology with human satellite III (ref. 5). *In situ* hybridization experiments have shown binding patterns to human, chimpanzee and orangutan chromosomes essentially identical to patterns shown by human satellite III (ref. 6).

Satellite DNAs have, for the most part, manifested a species specificity⁷⁻¹², although there is some evidence that not all satellite fractions do so. The best known exception is the polyd(A-T) fraction found in various crab and *Drosophila* species^{13,14}. Recently Graham and Skinner¹⁵ have demonstrated cross-hybridisation of a (G+C)-rich satellite in *Crustacea*, and Sutton and McCallum¹⁶ have shown similar results with the *Mus musculus* satellites and satellites from several Thailand species of *Mus*. The present report of cross-hybridisation in the higher primates is of special interest in that it demonstrates an inter-family hybridisation of a satellite sequence.

Abbreviation: SSC, 0.15 M NaCl-0.015 M sodium citrate

MATERIALS AND METHODS

DNA preparation

The frozen liver and spleen of a chimpanzee (*Pan troglodytes*) were used to prepare DNA according to the method of Marmur¹⁷. The molecular weight of the DNA so prepared was measured by sedimentation velocity in the Beckman analytical ultracentrifuge and found to be $2.7 \cdot 10^7$.

Preparative centrifugation in $\text{Ag}^+ - \text{Cs}_2\text{SO}_4$

The purified DNA was dissolved in 0.1 M Na_2SO_4 , and for centrifugation was used in a final concentration of 70 $\mu\text{g}/\text{ml}$. A solution of 0.1 M borate buffer (pH 9.2) was added to give a final concentration of 0.005 M with regard to the borate ion, and a 10^{-3} M solution of AgClO_4 was added to give a final Ag^+ to DNA phosphate (DNA-*P*) molar ratio (R_F) of 0.2. A saturated solution of Cs_2SO_4 , $\rho = 1.930 \text{ g}/\text{cm}^3$, (Merck Suprapur Cs_2SO_4 obtained from Anderman and Co., London) was added to a final density of 1.460–1.470 g/cm^3 as judged from the refractive index measurements.

Preparative centrifugation was carried out on volumes of 20 ml/tube in the 8×50 fixed angle rotor of the MSE 50 preparative ultracentrifuge. The preparation was spun at 30 000 rev./min, 25 °C for 96 h. 6-drop fractions (approx. 0.2 ml) were collected, diluted with 0.5 ml of 0.1 M Na_2SO_4 and their absorbance at 260 nm determined using the Unicam SP-800 spectrophotometer. The fractions pertaining to the region of satellite DNA were pooled and extensively dialyzed in 5 M NaCl plus 0.01 M Tris-HCl buffer (pH 7), and then dialyzed in $0.1 \times \text{SSC}$ (0.015 M NaCl–0.0015 M sodium citrate).

The chimpanzee satellite DNA in $0.1 \times \text{SSC}$ was concentrated by evaporation to approx. 30 $\mu\text{g}/\text{ml}$. CsCl (AnalaR, B.D.H. Chemicals, Poole, England) was added to give a final density of 1.710–1.720 g/cm^3 as judged from the refractive index, and the solution centrifuged in the 10×10 angle rotor of the MSE 50 preparative ultracentrifuge for 40 h at 42 000 rev./min. 4-drop fractions (approx. 0.13 ml) were collected, diluted with 0.5 ml $0.1 \times \text{SSC}$, and their absorbance at 260 nm determined in the Unicam SP-800 spectrophotometer.

Preparative centrifugation in $\text{Hg}^{2+} - \text{Cs}_2\text{SO}_4$

Preparative centrifugation of DNA in $\text{Hg}^{2+} - \text{Cs}_2\text{SO}_4$ was performed in a manner similar to the centrifugation of DNA in $\text{Ag}^+ - \text{Cs}_2\text{SO}_4$. The final concentration of DNA in 0.1 M Na_2SO_4 was 70 $\mu\text{g}/\text{ml}$, and borate was 0.005 M. A 10^{-3} M solution of HgCl_2 was added to give a final Hg^{2+} to DNA-*P* molar ratio of 0.10. Saturated Cs_2SO_4 was added to a final density of 1.45–1.47 g/cm^3 .

Centrifugation, collection of fractions, pooling and dialysis of fractions containing satellite DNA were carried out as described for $\text{Ag}^{2+} - \text{Cs}_2\text{SO}_4$ centrifugation.

Analytical centrifugation

Analytical centrifugation was carried out in the Beckman Model E analytical ultracentrifuge for 18 h at 44 000 rev./min and a temperature of 25 °C. Ultraviolet photographs were taken and traced on the Joyce-Loebel microdensitometer.

Samples of DNA with molar ratios of Ag^+ to DNA-*P* of 0.15, 0.25, 0.30 and

0.35 were tested for separation of the satellite fraction. In each case 70 $\mu\text{g/ml}$ of DNA was used and the amount of AgClO_4 solution added was adjusted. The amount of saturated Cs_2SO_4 added was adjusted according to the R_F used. Analytical ultracentrifugation of samples of DNA fractions obtained from $\text{Ag}^+-\text{Cs}_2\text{SO}_4$ and CsCl preparations was carried out in neutral CsCl (B.D.H. CsCl for ultracentrifugation). The densities of the DNAs were determined from the position of a marker DNA added to the gradients, in this case, *Micrococcus lysodeikticus* DNA, density equal to 1.731 g/cm^3 in neutral CsCl .

Samples of DNA in $\text{Hg}^{2+}-\text{Cs}_2\text{SO}_4$ were centrifuged using $R_F = 0.05, 0.15, 0.20$ and 0.25 . The amount of Hg^{2+} and Cs_2SO_4 were adjusted accordingly.

Human satellite III DNA

Human satellite III DNA was prepared from human placental tissue according to the method of Corneo *et al.*⁵.

Thermal denaturation profiles

Melting profiles of chimpanzee satellite A DNA were obtained on the Unicam SP-800 spectrophotometer fitted with a constant temperature cell housing. Temperature was monitored on a SP-876 temperature programme controller, raised at the rate of 1 $^\circ\text{C}/\text{min}$ and recorded intermittently on the read-out from a Unicam chart recorder. Melting was carried out in $0.1 \times \text{SSC}$. Graphs were plotted as a percentage increase in hyperchromicity.

Complementary RNA

Complementary RNA to chimpanzee satellite A and human satellite III DNA was made according to the method of Jones and Robertson¹⁸ using all four tritiated nucleotides. Specific activity of each complementary RNA was approx. 10^7 cpm/ μg .

Filter hybridisation

Filter hybridisation was carried out essentially according to the method of Birnstiel *et al.*¹⁹. Gradients of chimpanzee, human and rat DNA were prepared in the following way. High molecular weight DNA was dissolved in $0.1 \times \text{SSC}$ at a concentration of approx. 50 $\mu\text{g/ml}$. CsCl (1.28 g/ml) was added to form an initial buoyant density of 1.700 g/ml . Tubes containing 4 ml of this solution were centrifuged to equilibrium in a 10×10 MSE angle rotor at 25 $^\circ\text{C}$ and 42 000 rev./min for 42 h. The tubes were punctured and 10-drop fractions were collected. These fractions were diluted with 0.5 ml $0.1 \times \text{SSC}$ and their absorbance read at 260 nm. They were then denatured by the addition of an equal volume of 1.0 M NaOH for 15 min at room temperature, neutralised with neutralising mix (1.0 M HCl, 1.0 M Tris buffer (pH 8.0), 3.0 M NaCl (1:1:2, by vol.) added at 4 times the initial volume, and allowed to drip through individual 13-mm Millipore filters (HAWP 0.45 μm pore size) which had been prewashed in $2 \times \text{SSC}$. The loaded filters and blanks were washed with $6 \times \text{SSC}$, using approx. 2 ml per filter, dried in a vacuum oven at 80 $^\circ\text{C}$ for 2 h, labelled and stored until use at -20°C .

Hybridisation was performed with chimpanzee satellite A complementary RNA and human satellite III complementary RNA to filters loaded with human, chimpanzee and rat DNA. The filters were incubated in 0.5 ml of $2 \times \text{SSC}$ containing

5 μ l of radioactive complementary RNA (a total of $1.1 \cdot 10^5$ – $1.2 \cdot 10^5$ counts) at 68 °C for 4 h. They were then chilled, washed in 1 change of $6 \times$ SSC and 3 changes of $2 \times$ SSC, ribonucleased for 6 min (10 μ g/ml ribonuclease in $2 \times$ SSC), dried and counted in 10 ml of toluene based scintillation fluid in a Nuclear Chicago scintillation counter. Machine counts were deducted.

RESULTS

Ag^+ – Cs_2SO_4 centrifugation of chimpanzee DNA

When chimpanzee DNA was centrifuged in Ag^+ – Cs_2SO_4 gradients with varying molar ratios of Ag^+ to DNA-*P*, one satellite was observed. Its position varied according to the amount of Ag^+ used. Fig. 1 shows these results.

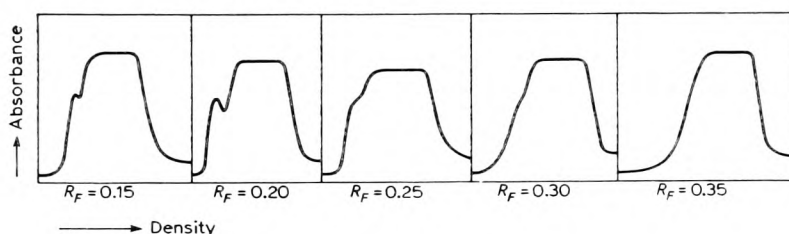


Fig. 1. Analytical equilibrium density gradient centrifugation of chimpanzee total DNA in Ag^+ – Cs_2SO_4 . The conditions of centrifugation are described in the text. The initial buoyant densities of the five samples were respectively 1.460, 1.471, 1.482, 1.493, 1.504 g/cm³.

A molar ratio of 0.20 was used in the preparation of chimpanzee satellite DNA. Fig. 2 shows the pattern obtained in fractionation of a preparative gradient, together with the analytical tracing of a sample from the same preparation.

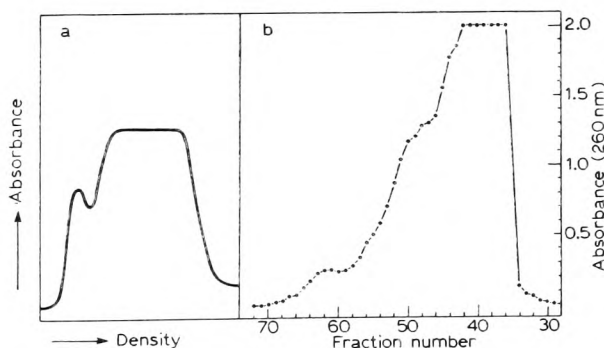


Fig. 2. Analytical (a) and preparative (b) equilibrium density gradient centrifugation of chimpanzee total DNA in Ag^+ – Cs_2SO_4 , $R_F = 0.20$. The conditions of preparation are reported in the text. The small peak corresponds to satellite DNA, the large peak to mainband DNA.

The fractions obtained were dialyzed against 5 M NaCl and $0.1 \times$ SSC as described, then centrifuged in neutral CsCl in the analytical centrifuge. Fig. 3 shows the analytical tracings of six of these fractions from various positions across the gradient.

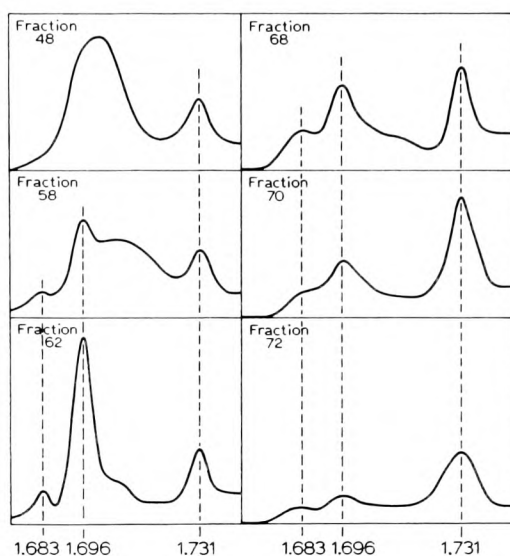


Fig. 3. Microdensitometer tracings of fractions of chimpanzee DNA obtained from preparative centrifugation in the Ag^+ - Cs_2SO_4 density gradient, centrifuged in neutral CsCl in the analytical ultracentrifuge. The fraction numbers correspond to those in Fig. 2b. The peak of buoyant density = 1.731 g/cm^3 corresponds to the marker DNA.

Fractions 60–70 of Fig. 2b were pooled, concentrated, and centrifuged in CsCl as described in Materials and Methods. Fig. 4 shows these results.

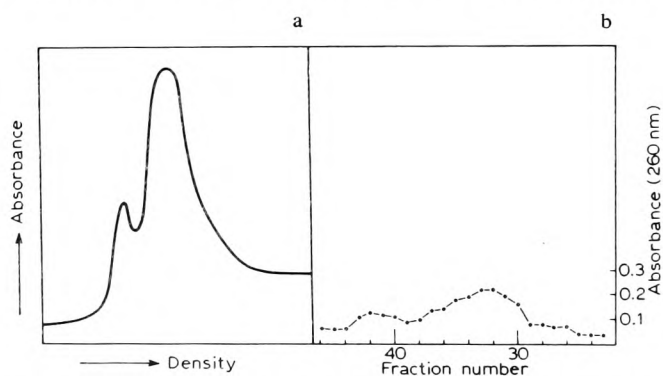


Fig. 4. Analytical (a) and preparative (b) equilibrium density gradient centrifugation of chimpanzee satellite DNA obtained from the Ag^+ - Cs_2SO_4 gradient (Fig. 2b) and recentrifuged in CsCl as described in the text. The small peak corresponds to satellite B, the major peak to satellite A. Mainband DNA with a density of 1.700 g/cm^3 is not shown.

The fractions obtained were dialyzed against $0.1 \times \text{SSC}$ then centrifuged in neutral CsCl in the analytical ultracentrifuge. Fig. 5 shows the analytical tracings of several of these fractions.

The single satellite peak seen in the Ag^+ - Cs_2SO_4 gradient (Fig. 2) is observed to break into two distinct satellites in CsCl centrifugation (Fig. 4), satellite A with a buoyant density of 1.696 g/cm^3 in neutral CsCl and satellite B with a buoyant density

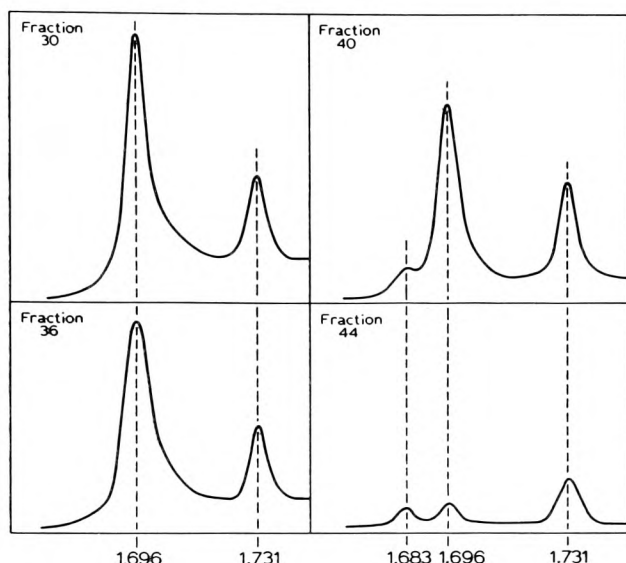


Fig. 5. Microdensitometer tracings of fractions of chimpanzee satellite DNA obtained from preparative centrifugation of chimpanzee Ag^+ - Cs_2SO_4 satellite in CsCl . The fraction numbers correspond to those in Fig. 3b. The peak of buoyant density 1.731 g/cm^3 corresponds to the marker DNA.

of 1.683 g/cm^3 . Chimpanzee mainband DNA has a buoyant density of 1.700 g/cm^3 in neutral CsCl .

Although Fig. 4 implies a separation of satellites A and B, analytical inspection of the gradient (Fig. 5) shows that the smaller peak, satellite B, is continuously contaminated by satellite A. A similar inspection of the gradient in Fig. 2 (Fig. 3) shows that peaks of DNA of the same buoyant density as the satellite fractions persist in fractions well into the mainband. Because a complete separation of satellites from mainband and from each other is not obtained, the percentage of the genome comprised by the satellites in chimpanzee cannot be judged from analytical tracings. The reason for this incomplete separation is unclear. It may be due to progressively lower molecular weight DNA being recovered from the procedure or it may be that satellite B is a very small proportion of the genome and sufficient DNA to be optically visible is not present on the extreme light side of the gradient. Satellite A can be obtained in pure form from suitable fractions of the Ag^+ - Cs_2SO_4 or the CsCl gradient. No other satellites were observed. Fractions 32 and 34 from the extreme heavy side of the gradient in Fig. 2 contained only mainband DNA.

Comparison of chimpanzee satellite A and human satellite III

The buoyant density of native satellite A is 1.696 g/cm^3 of denatured satellite is 1.713 g/cm^3 , and of renatured satellite is 1.706 g/cm^3 . When centrifuged in alkaline CsCl it separates into two strands of buoyant densities 1.791 g/cm^3 and 1.755 g/cm^3 . (Fig. 6.)

The physical characteristics of chimpanzee satellite A are very similar to those of human satellite III which has a native buoyant density of 1.696 g/cm^3 , denatured buoyant density of 1.715 g/cm^3 , and a renatured (60°C , 5 h in $2 \times \text{SSC}$) buoyant

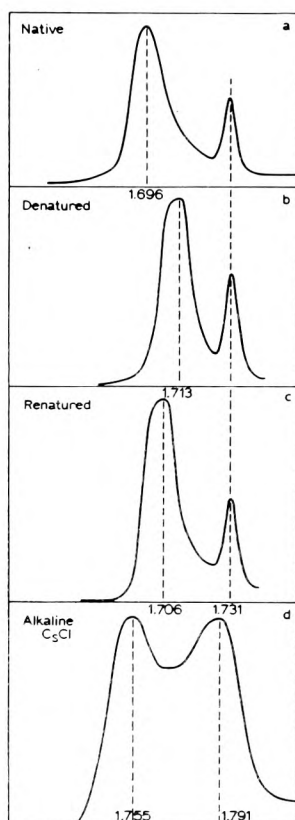


Fig. 6. Microdensitometer tracings of chimpanzee satellite DNA (A) centrifuged to equilibrium in the analytical ultracentrifuge in neutral CsCl (a) native (b) denatured by heating at 100 °C for 10 min in $0.1 \times$ SSC followed by rapid cooling (c) renatured at $10 \mu\text{g/ml}$ at 65 °C for 5 h in $2 \times$ SSC after heat denaturation as in (b). Separation of strands in alkaline CsCl is shown in (d). This was achieved by adding 0.025 ml of M NaOH to $15 \mu\text{g}$ of DNA in 0.8 ml $0.1 \times$ SSC. CsCl was added to a final density of 1.755–1.770 g/cm^3 . Densities in neutral CsCl were determined from *Micrococcus lysodeikticus* DNA, density = 1.731 g/cm^3 , and in alkaline CsCl from the limiting isoconcentration distance and the initial density of the solution.

density of 1.703 g/cm^3 . Human satellite III separates in alkaline CsCl into strands of buoyant densities 1.754 g/cm^3 and 1.740 g/cm^3 (ref. 5).

The T_m (mean melting temperature, defined as the temperature at which 50 % of the molecules are single stranded) of native chimpanzee satellite A is 69 °C and of renatured satellite is 59 °C giving a ΔT_m of 10 °C.

If the effect of single strand hyperchromicity is taken into consideration when viewing the melting curve of renatured chimpanzee satellite, a slightly different interpretation is afforded. This can be done in the following way: From the equation $32a + 5.3(1 - a) = 18$, where a is the proportion of DNA in duplex form, 32 is the percent hyperchromicity obtained for native duplex chimpanzee satellite A DNA, 5.3 is the percent hyperchromicity above 45 °C for single-stranded DNA, and 18 is the percent hyperchromicity for "renatured" chimpanzee satellite DNA containing a mixture

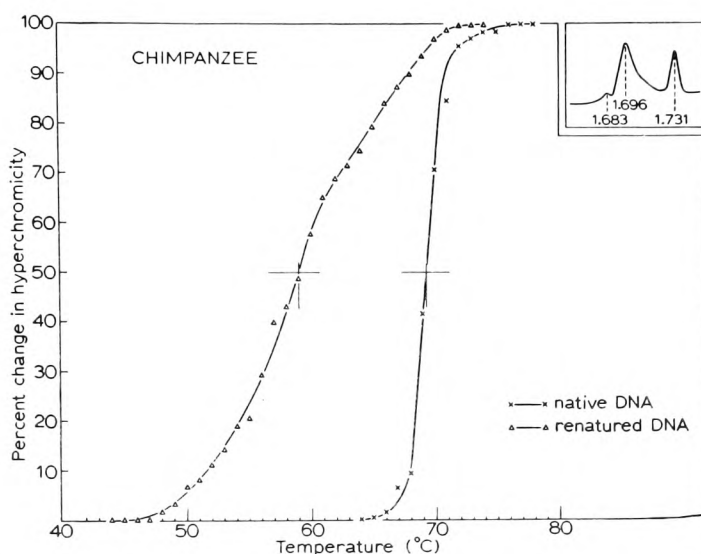


Fig. 7. The melting curves of native and renatured chimpanzee satellite (A) DNA in $0.1 \times \text{SSC}$. The renatured satellite sample was first denatured in $0.1 \times \text{SSC}$ at 100°C for 10 min, then made up to $2 \times \text{SSC}$ and renatured for 5 h at $20 \mu\text{g/ml}$ at 65°C . It was then dialyzed into $0.1 \times \text{SSC}$ before melting. Total percent hyperchromicity for native satellite was 32 %, for renatured satellite was 18 %. The microdensitometer tracing in the upper right hand corner shows that the samples consisted mainly of satellite A with a slight contamination of satellite B.

of single stranded and duplex molecules, it is calculated that 15.5 % of the hyperchromicity indicated on the graph is due to single stranded hyperchromicity. The T_m calculated in this way is 60°C , giving a ΔT_m of 9°C .

The biphasic character of the renatured melt indicates that two populations of molecules are present, one more homogeneous than the other.

Human satellite III in our hands gave a less sharp native melting curve than chimpanzee satellite A, indicating a more heterogeneous population of molecules. The T_m was 69°C in $0.1 \times \text{SSC}$. Corneo *et al.*⁵ carried out thermal denaturations in SSC on human satellite III DNA, native and renatured (C_0t of approx. 2). (We find approximately 15°C difference between melts performed in SSC and melts in $0.1 \times \text{SSC}$.) Corneo found the native T_m was equivalent to 69°C in $0.1 \times \text{SSC}$. His remelting curve shows a long "foot", probably due to hyperchromicity of single-stranded molecules, and a sharp thermal transition, probably due to renatured duplex, with a T_m equivalent to 62°C , giving a ΔT_m of 7°C .

Table I summarizes the physical properties of the two similar satellite fractions from chimpanzee and human.

Hg^{2+} - Cs_2SO_4 centrifugation of chimpanzee DNA

Several molar ratios of Hg^{2+} to DNA-P were tested on chimpanzee DNA. Results comparable to those for Ag^+ - Cs_2SO_4 shown in Fig. 1 were obtained. A molar ratio of 0.05 in a gradient with $70 \mu\text{g/ml}$ was found to give the best separation of satellites, indicating the presence of two satellite fractions, one on the light side of the gradient, and one on the heavy side.

TABLE I
PHYSICAL PROPERTIES OF CHIMPANZEE SATELLITE A AND HUMAN SATELLITE III

| | Buoyant density | T_m (°C) | % increase in hyperchromicity |
|-------------------------------|-----------------|------------|-------------------------------|
| <i>Chimpanzee satellite A</i> | | | |
| Native | 1.696 | 69 | 32 |
| Denatured | 1.713 | | |
| Renatured | 1.706 | 60 | 18 |
| <i>Human satellite III</i> | | | |
| Native | 1.696* | 69** | 30 |
| Denatured | 1.715* | | |
| Renatured | 1.703* | 62** | — |

* Taken from Corneo *et al.*⁵.
** Derived from Corneo *et al.*⁵.

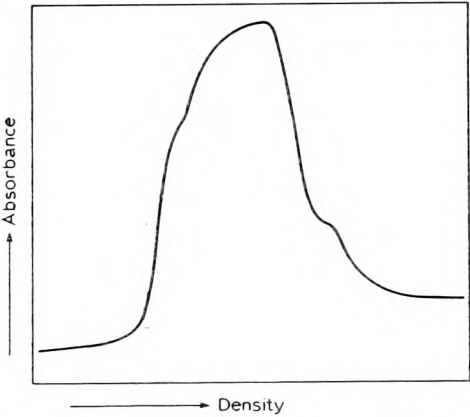


Fig. 8. Analytical equilibrium density gradient centrifugation of chimpanzee total DNA in Hg^{2+} - Cs_2SO_4 , $R_F = 0.05$. The conditions of centrifugation are described in the text. Satellite A is present as a shoulder on the light side of the gradient, satellite B is shown as a peak on the heavy side of the mainband.

Fig. 8 shows the presence of these two satellite fractions in addition to the mainband DNA. Fractions containing the two satellites were isolated from a preparative gradient and were shown to have the same buoyant densities as the satellites from the Ag^+ - Cs_2SO_4 gradient.

Centrifugation of chimpanzee total DNA in Hg^{2+} - Cs_2SO_4 gradients did not succeed in showing the presence of more or different chimpanzee satellite DNAs. It did, however, show a different binding pattern of heavy metal ions to the same satellite fractions. Satellites A and B separated in the Hg^{2+} - Cs_2SO_4 gradient, satellite A being found on the light side, as in Ag^+ - Cs_2SO_4 centrifugation, satellite B being found on the heavy side, unlike the situation in Ag^+ - Cs_2SO_4 where both are present on the light side. Satellite B was not obtained free from mainband contamination in Hg^{2+} - Cs_2SO_4 .

Filter hybridisation

Chimpanzee satellite A and human satellite III have similar physical properties. Our filter hybridisation results indicate that this similarity extends to shared nucleotide sequences.

Complementary RNAs transcribed from each of the satellite fractions hybridised to their homologous DNAs at buoyant densities corresponding to native satellite DNA, *i.e.* 1.696 g/cm^3 (Figs 9 and 10). The heterologous reactions in each case indicated hybridisation to the same buoyant density fractions, but to a lesser extent. Hybridisation to control DNA (rat) did not occur above background counts and is not illustrated. It is concluded that human satellite III and chimpanzee satellite A share a proportion of their highly repetitive sequences. Data (Jones, K. W., unpublished) indicate the shared homology is approx. 25–30%.

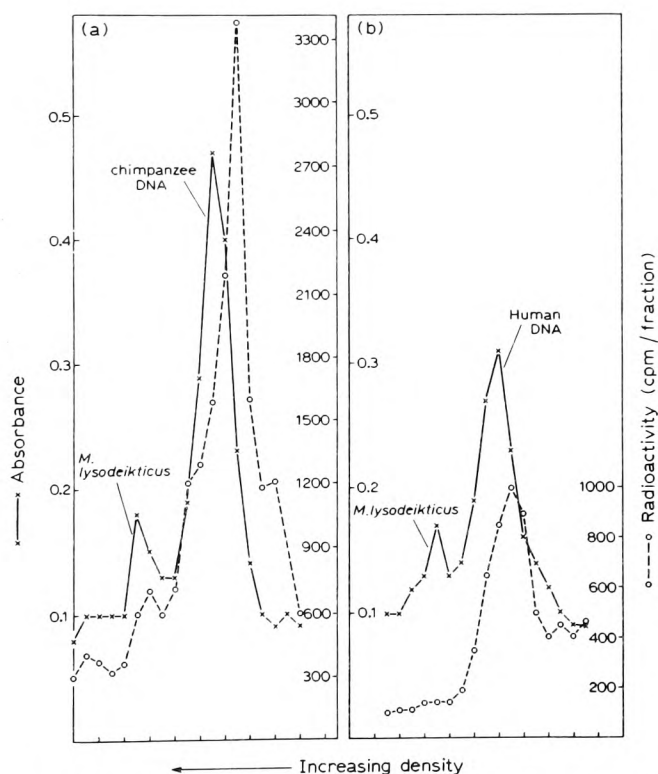


Fig. 9. Filter hybridisation results of chimpanzee satellite A complementary RNA to gradients of chimpanzee DNA (a) and human DNA (b). *M. Lysodeikticus* DNA, buoyant density = 1.731 g/cm^3 was used as a marker. The method is described in the text. $\times - \times$, A_{260} of fractionated DNA; $\circ - \circ$, cpm per fraction of complementary RNA hybridised to DNA.

DISCUSSION

Chimpanzee DNA banded in CsCl in normal loadings of $4\text{--}5 \mu\text{g/ml}$ or in overloadings of $25\text{--}30 \mu\text{g/ml}$ does not separate into satellite and mainband fractions.

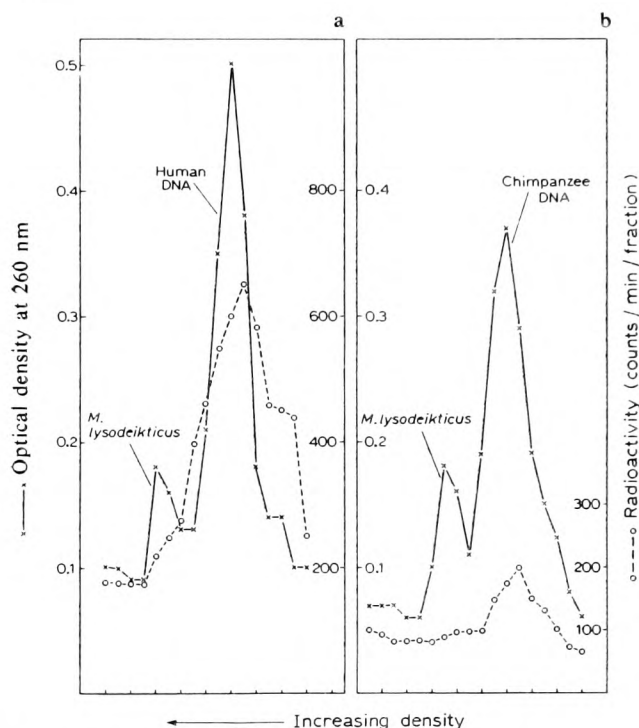


Fig. 10. Filter hybridisation results of human satellite III complementary RNA to gradients of human (a) and chimpanzee DNA (b). *M. Lysodeikticus* DNA, buoyant density = 1.731 g/cm³, was used as a marker. The method is described in the text. $\times - \times$, $A_{260 \text{ nm}}$ of fractionated DNA; $\circ - \circ$, cpm per fraction of complementary RNA hybridised to DNA.

However, in the presence of heavy metal ions, Ag^+ and Hg^{2+} , we have demonstrated two satellite fractions in Cs_2SO_4 gradients. These (A+T)-rich fractions do not bind heavy metal in a predictable way: satellite B (1.683 g/cm³) is found on the light side of the mainband in the Ag^+ - Cs_2SO_4 gradient, and on the heavy side in the Hg^{2+} - Cs_2SO_4 gradient. Satellite A on the other hand remains on the light side in both types of gradient.

Satellite A was isolated and characterised and found to be similar to human satellite III in its physical properties. This similarity extends to the existence of underlying base sequence homology as was shown by hybridisation of complementary RNAs to the two satellites against total human and chimpanzee DNA on filters. Cytological cross hybridisation of the respective complementary RNAs to chromosomes of chimpanzee, orangutan and human showed similar site specificity to that obtained in homologous hybridisations⁶.

The cross hybridisation results of the two satellites indicate a common origin for them in a primitive ancestral form. The ΔT_m data (9 °C for chimpanzee and 7 °C for human) are compatible with this, since they infer similar average extents of nucleotide change. Kohne²⁰ has calculated a 2.5 % change in unique copy DNA between man and chimpanzee. It is evident that the satellite DNAs which we describe are considerably more diverged. Whether the satellite DNA has diverged more rapidly

than unique copy DNA or whether the satellite occurred before divergence of the two species remains to be determined. Examination of relatives of man and chimpanzee which diverged earlier may shed light on the time of origin of this sequence. Chimpanzee B satellite (1.683 g/cm^3) has a buoyant density similar to the human satellite I (1.687 g/cm^3) (ref. 21) and like satellite I is found on the heavy side of the mainband in Hg^{2+} - Cs_2SO_4 gradients and on the light side of the mainband in Ag^+ - Cs_2SO_4 gradients. This satellite bands on the light side of satellite A in the same way that human satellite I bands on the light side of III in Ag^+ - Cs_2SO_4 gradients. We are investigating these two satellites to see if they share common sequences.

The ΔT_m of satellite I (ref. 22) indicates that it is a younger satellite than human III and chimpanzee A. From its melting properties, human satellite II (ref. 23) is even younger than satellite I. We have not yet examined the chimpanzee for the presence of this satellite by hybridisation methods, but so far the centrifugation techniques used here have failed to reveal a similar buoyant density fraction in the chimpanzee.

ACKNOWLEDGEMENTS

We wish to thank Prof. M. Birnstiel and J. Speirs for their help in the preparation of this work, also Mr E. D. Roberts for drawing the graphs presented here. *M. lysodeikticus* DNA was a gift from J. Speirs.

J. P. was supported by a Muscular Dystrophy Association of Canada fellowship during the period of this work, and was also in receipt of a Wellcome Foundation travel grant to visit the laboratory of Prof. G. Corneo, Milan, Italy. The studies presented are in partial fulfillment of the requirements for a Ph. D. degree.

This work was supported by grants from the Medical Research Council and the Muscular Dystrophy Group of Great Britain to K. W. J.

REFERENCES

- 1 Britten, R. J. and Kohne, D. F. (1968) *Science* 161, 529-540
- 2 Walker, P. M. B. (1971) in *Progress in Biophysics and Molecular Biology* (Butler, J. A. V. and Noble, D. eds), Vol. 23, pp. 143-190, Pergamon Press, Oxford, and New York
- 3 Coudray, Quetier, F. and Guille, E. (1970) *Biochim. Biophys. Acta* 217, 259-267
- 4 Arrighi, F. E., Mandel, M., Bergendahl, J. and Hsu, T. C. (1970) *Biochem. Gen.* 4, 367-376
- 5 Corneo, G., Ginelli, E. and Polli, E. (1971) *Biochim. Biophys. Acta* 247, 528-534
- 6 Jones, K. W., Prosser, J., Corneo, G., Ginelli, E. and Bobrow, M. (1972) in *Constitutive Heterochromatin in Man*, Symposia Medica Hoechst, Schattauer Verlag, Stuttgart, in the press
- 7 Walker, P. M. B., Flamm, W. G. and McLaren, A. (1969) in *Handbook of Molecular Cytology* (Lima-de-Faria, A. ed.), pp. 52-66, North Holland, Amsterdam and London
- 8 Gall, J. G., MacGregor, H. C. and Kidson, M. E. (1969) *Chromosoma Berlin* 26, 169-187
- 9 Gall, J. G. (1970) *J. Cell Biol.* 47, 68a
- 10 Gall, J. G., Cohen, E. H. and Polan, M. L. (1971) *Chromosoma Berlin* 33, 319-344
- 11 Mazrimas, J. A. and Hatch, F. T. (1972) *Nat. New Biol.* 240, 102-105
- 12 Gibson, I. and Hewitt, G. (1972) *Chromosoma Berlin* 38, 121-138
- 13 Skinner, D. M. and Kerr, M. S. (1970) *J. Cell Biol.* 47, 513a
- 14 Fransler, B., Travaglini, E., Loeb, L. and Schultz, J. (1970) *Biochem. Biophys. Res. Commun.* 40, 1266-1272
- 15 Graham, D. E. and Skinner, D. M. (1973) *Chromosoma Berlin* 40, 135-152
- 16 Sutton, W. D. and McCallum, M. (1972) *J. Mol. Biol.* 71, 633-656

- 17 Marmur, J. (1961) *J. Mol. Biol.* 3, 208–218
- 18 Jones, K. W. and Robertson, F. W. (1970) *Chromosoma Berlin* 31, 331–345
- 19 Birnstiel, M. L., Speirs, J., Purdom, I., Jones, K. and Loening, U. E. (1968) *Nature* 219, 454–463
- 20 Kohne, D. E. (1970) *Q. Rev. Biophys.* 33, 327–375
- 21 Corneo, G., Ginelli, E. and Polli, E. (1967) *J. Mol. Biol.* 23, 619–622
- 22 Corneo, G., Ginelli, E. and Polli, E. (1968) *J. Mol. Biol.* 33, 331–335
- 23 Corneo, G., Ginelli, E. and Polli, E. (1970) *J. Mol. Biol.* 48, 319–327

The Chromosomal Location of Human Satellite DNA III

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Abstract. *In situ* hybridisation of radioactive complementary RNA has been used to localise the chromosomal distribution of human satellite DNA III. This DNA is found to be concentrated in paracentromeric heterochromatin mainly on chromosome 9 and in minor concentrations on chromosomes chiefly of the D and G groups.

Introduction

Human DNA has been shown to contain at least four well authenticated satellite DNA fractions in isopycnic CsCl gradients. Satellite I (1.687 g/cm^3) (Corneo *et al.*, 1968); satellite II (1.693 g/cm^3) (Corneo *et al.*, 1970); satellite III (1.696 g/cm^3) (Corneo *et al.*, 1971) and satellite IV (Corneo *et al.*, 1972). These constitute respectively about 0.5, 2.0, 1.5 and 2.0% of the total genomal DNA. The chromosomal location of satellites I and IV is as yet undetermined, but that of satellite II has recently been shown by the method of *in situ* hybridisation (Jones and Corneo, 1971). In the present communication we describe the location of satellite III DNA as determined by *in situ* hybridisation of complementary RNA to chromosomes and nuclei of human peripheral blood lymphocytes.

Materials and Methods

Human total DNA was obtained by the method of Marmur (1961) from human spleen and placenta. The method of isolation and purification of satellite DNA fractions by silver caesium sulphate gradient centrifugation was that described by Corneo *et al.* (1971). $6 \mu\text{g}$ of satellite DNA were transcribed into c-RNA as follows: approximately equimolar (0.4 mM) amounts of each of the tritium labelled nucleoside triphosphates ATP (22.7 Ci/mM), UTP (14.0 Ci/mM), CTP (20.8 Ci/mM) and GTP (9.9 Ci/mM) in solution as received from Radiochemicals (Amersham), together with the DNA in water were freeze dried in the bottom of a 10 ml conical centrifuge tube. To this was added 0.3 ml of 0.1 M Tris-HCl buffer (pH 7.9 at 30°C) containing 1.6 mM spermidine, $0.1 \mu\text{l}$ mercaptoethanol ($1 \text{ ml} = 1.1168 \text{ g}$; Sigma), 2.5 mM MnCl_2 and 5 units of *E. coli* RNA polymerase previously prepared according

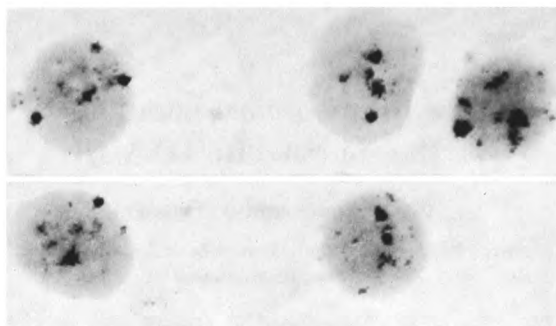


Fig. 1. Interphase nuclei after hybridisation *in situ* with human satellite III complementary RNA. In each nucleus there are two prominent black areas which represent the masses of autoradiographic grains over the centromeric heterochromatin of chromosomes number 9. Subsidiary black areas are those due to minor chromosomal regions which have also hybridised. It is clear from these grain distribution patterns that the chromosomal regions which hybridise with satellite III complementary RNA remain condensed

to the method of Chamberlain and Berg (1961). The reaction was carried out for 90 min at 30° C. After incubation 5 mM MgCl₂ and 40 µg/ml RNAase-free DNAase (Worthington) were added and digestion carried out for 10 min at 37° C. Non-radioactive *E. coli* RNA (500 µg) was then added as carrier and the mixture made 0.2% in sodium lauryl sulphate and 0.1 M in NaCl. After 2 min at 37° C, the mixture was shaken with an equal volume of water-saturated phenol for 10 min at room temperature. After 10 min of centrifugation at 10000 r.p.m. in a Sorval RC2B centrifuge the upper aqueous layer was removed and set aside. The phenol was extracted with an equal volume of water and recentrifuged. The aqueous layer was then added to that from the previous step. The combined extract was applied to a 1.7 × 30 cm column of "Sephadex" G50 equilibrated with 0.1 SSC (SSC = 0.15 M NaCl, 0.015 trisodium citrate, pH 7.2). The column was developed with 0.1 SSC and 0.5 ml fractions collected. The radioactive excluded fractions containing carrier RNA and c-RNA were pooled, lyophilised, and re-dissolved in 4 × SSC allowing for salt already present. The final specific activity was estimated to be 1.1×10^8 DPM/µg. Before use in *in situ* experiments its hybridisation specificity for human DNA was tested by hybridisation to filters loaded with either human or mouse total DNA. *In situ* hybridisation of the c-RNA to air-dried chromosome preparations of phytohaemagglutinin-stimulated human peripheral lymphocytes was then carried out as follows: Slides were incubated at room temperature in 0.2 N HCl to denature the DNA *in situ*. The denatured preparations were dehydrated through an alcohol series and air-dried. Approximately 5 µl of c-RNA was placed on each preparation which was then covered with a 22 mm square coverslip and sealed with rubber solution. The slides were then incubated on a steel tray in a covered water bath at 65° C for 10 hours, after which they were rapidly cooled on ice and the coverslips detached. The c-RNA was then removed by washing in 2 × SSC at room temperature and non-specific complexes removed by RNAase treatment (20 µg/ml at 37° C for 20 min). The slides were then washed in two further baths of 2 × SSC and finally washed for 4 hours in a stirred bath

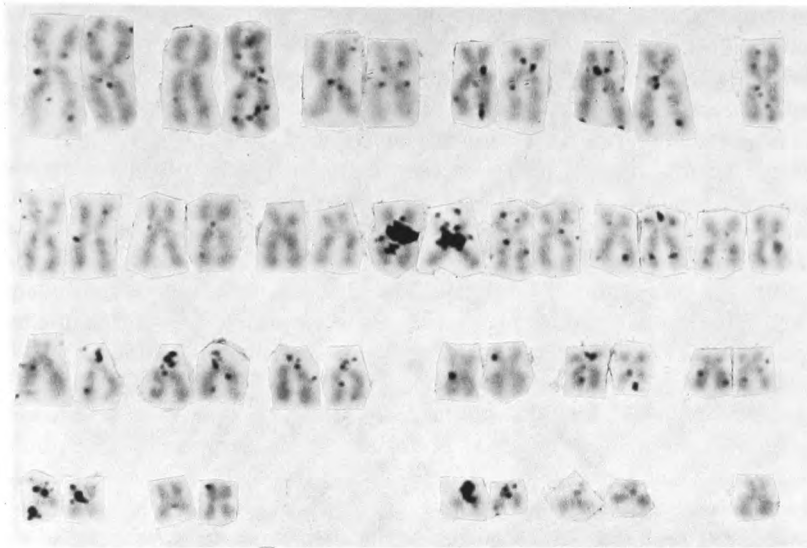


Fig. 2. Karyotype of metaphase chromosomes after hybridisation with satellite III complementary RNA. The two chromosomes with prominent masses of autoradiographic grains over the centromeric regions are the pair number 9. Other minor regions of hybridisation are evident on other chromosome groups, particularly the acrocentrics of the D and G groups

containing 2 litres of $2 \times$ SSC before being dehydrated through an alcohol series and air dried. Control slide preparations of mouse foetal fibroblast chromosomes were used. Hybridised slides were coated with Ilford K-2 emulsion diluted 1:1 with water and exposed in plastic boxes at 4°C for 4–10 weeks. Development was for $3\frac{1}{2}$ min in D 19. The preparations were stained in toluidine blue and photographed on Pan F film using a Zeiss Universal microscope.

Results and Discussion

Mouse nuclei and chromosomes hybridised with human satellite III complementary RNA showed only sparse, non-specific autoradiographic grains. Human interphase nuclei, by comparison, showed a consistent pattern of silver grains with two main locations which appeared as black spots due to high local concentrations of radioactivity and consequent saturation of the emulsion (Fig. 1). In nuclei entering prophase and in arrested metaphases these two main concentrations were associated with the long arms of chromosome pair 9 close to the centromeres (Fig. 2). The identical pattern of grains at metaphase and interphase indicated that the sequences hybridised are also localised in condensed

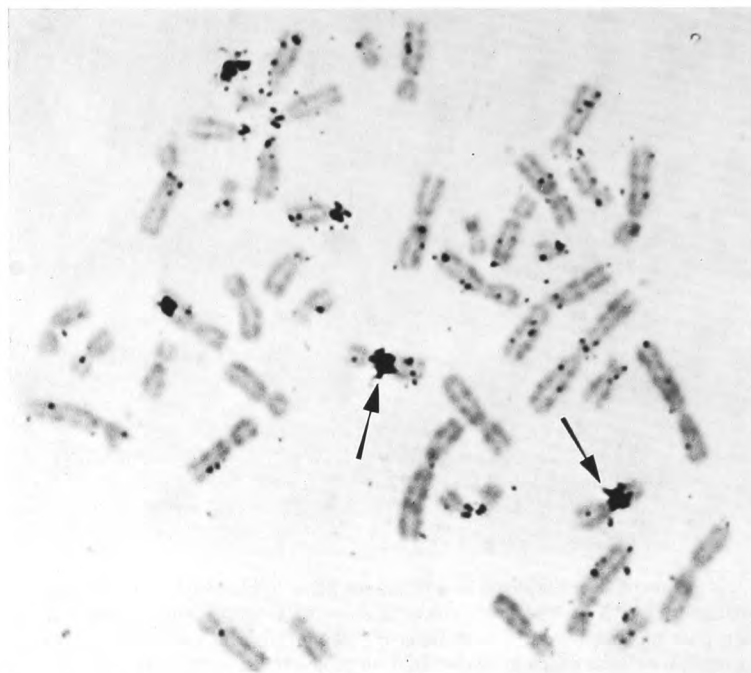


Fig. 3. Metaphase chromosomes from an individual heterozygous for a pericentric inversion of the prominent constitutive heterochromatin block on chromosome 9 (arrows). In this case the pattern of autoradiographic grains is different on the homologues of pair 9; being on the short arm of one (inversion) and on the long arm of the other. In other respects the distribution of grains is similar to that of Fig. 2

chromatin in interphase. Satellite III sequences are known from chromatin fractionation studies to be concentrated within dense chromatin (Corneo *et al.*, 1971). Chromosome 9 is known to have a prominent heterochromatic region at this location which stains intensely with Giemsa both in interphase and metaphase (Arrighi and Hsu, 1971; Bobrow *et al.*, 1972). The involvement of chromosome 9 was established by the use of a karyotype containing a pericentric inversion of the centromeric heterochromatin on this chromosome. In this case the region hybridised was seen to be present next to the centromere on the short arm of one of the homologues, and on the long arm of the other (Fig. 3). We conclude that the bulk of the satellite III DNA is extracted from this region on the pair of chromosomes number 9. There are minor locations of hybrids in interphase nuclei, however, which suggest that this sequence is not exclusive to chromosome 9 and inspection of meta-

phase chromosomes after 10 weeks exposure revealed a small number of grains in association with the constitutive heterochromatin near the centromeres of the chromosomes of groups D and G. The centromeres of chromosome 16 and those of chromosome pair 1, very occasionally, show one or two grains. In previous studies of localisation of satellite II sequences a similar distribution was found in respect of the minor locations but in this case chromosomes 1 and 16 hybridised strongly and chromosome 9 less so. Chromosomes 1, 9 and 16 therefore contain the bulk of the satellite sequences so far discovered in human DNA, including a recently reported satellite DNA (Saunders *et al.*, 1972) which hybridises *in situ* with the same region of chromosome 9. This latter satellite DNA may be identical with satellite III (Corneo *et al.*, 1971) or the newly identified satellite IV (Corneo *et al.*, 1972). The possibility of cross-contamination of satellites II and III as an explanation of their overlapping hybridisation patterns is very unlikely since these fractions band on opposite sides of the mainband DNA in the conditions of $\text{Ag}^+\text{Cs}_2\text{SO}_4$ used for their preparation, and they were shown to be pure by analytical ultracentrifugation of the template DNA used in the preparation of the radioactive complementary RNA. Moreover, a level of contamination of III with II high enough to register in minor locations on the D and G group would be expected to show strong location on the chromosomes 1 and 16 and this was not observed. We therefore conclude that human constitutive paracentromeric heterochromatin is composed of a number of distinct repetitive DNA sequences. The relative proportions of each sequence however, vary and are characteristic of given chromosomes. In a previous communication (Jones and Corneo, 1971) it was shown that a major satellite-containing region on chromosome 1 was structurally polymorphic in certain individuals such that one homologue contained significantly less satellite DNA than the other homologue. In the present instance we show heterozygosity for a structural rearrangement of a satellite DNA-rich region on chromosome 9. This rearrangement is visible after Giemsa staining and has been described by Lubs and Ruddle (1970a) and by Bobrow *et al.* (1972). It seems that such variations in chromosome structure in otherwise normal individuals may be quite widespread in the population (Craig-Holmes *et al.*, 1972). The frequency of certain variants, particularly those associated with the D and G group, is statistically different in Negro and Caucasian populations (Lubs and Ruddle, 1971) and the presence of certain of the rare variants may also carry an increased risk of phenotypic abnormality in Caucasian infants (Lubs and Ruddle, 1970b).

The pattern of grains over interphase nuclei indicated, at least in peripheral blood lymphocytes, that the heterochromatin of chromosome

pair 9 forms separate chromocentres. Other minor locations may sometimes however be seen to participate in the formation of a more loosely organised chromocentre separate from the other two. This observation is consistent with the tendency of telocentric chromosomes to exhibit centric associations and may reflect some common functional activity, for example nucleolus organisation. It seems likely that such shared functions involving physical contacts, may, in the course of evolution, tend to favour the spread of centromeric heterochromatin and its associated satellite DNA among certain non-homologous chromosomes. Whether such a mechanism might also potentiate the evolution of metacentric chromosomes by Robertsonian fusion is an interesting speculation at present. An extreme form of such non-random distribution of satellite DNA, perhaps correlating with common chromosomal functions, has been shown in the Japanese quail (*Coturnix coturnix japonica*) in which the heavy satellite DNA is localised exclusively in the microchromosomes which form a chromocentre at interphase (Brown and Jones, 1971).

Satellite III DNA shows more sequence divergence than does satellite II DNA as measured by their renaturation behaviour (Corneo *et al.*, 1971; Prosser, Moar, Bobrow and Jones, in press 1973). This suggests that satellite III is older in evolution than satellite II. We have recently shown that satellite III cross hybridises *in situ* with certain paracentromeric regions of chimpanzee and orang-utan chromosomes (Jones *et al.*, 1972) and that a physically similar partly homologous satellite DNA is present in the chimpanzee (Prosser, Moar, Bobrow and Jones, in press 1973). Radioactive c-RNA made on a template of the chimpanzee satellite hybridises with human chromosome 9 so that it appears that the time of origin of the original satellite III sequence pre-dates the evolutionary divergence of man, orang-utan and chimpanzee. Satellite II however appears absent from the chimpanzee (unpublished observation). These observations should enable us to reconstruct the likely phylogeny of the higher apes since it appears that so far they may be unique in possessing common satellite DNA's.

Acknowledgements. The authors gratefully acknowledge the following financial support during the period of this work; The Medical Research Council, Cancer Campaign, and Muscular Dystrophy Group of Great Britain (K.W.J.); the Consiglio Nazionale delle Ricerche (Rome) Grant No. 71.00802.04.115.2155 (G.C.); the Muscular Dystrophy Association of Canada (J.P.). J.P. also wishes to thank the Wellcome Foundation for a travel grant to visit the laboratory of G.C. We thank Dr. Martin Bobrow for providing chromosome preparations showing pericentric inversion of chromosome pair 9.

References

- Arrighi, F. E., Hsu, T. C.: Localisation of heterochromatin in human chromosomes. *Cytogenetics* **10**, 81-86 (1971).
- Bobrow, M., Madan, K., Pearson, P. L.: Staining of some specific regions of human chromosomes particularly the secondary constriction of No. 9. *Nature (Lond.) New Biol.* **238**, 122-124 (1972).
- Brown, J. E., Jones, K. W.: Localisation of satellite DNA in the microchromosomes of the Japanese quail by in situ hybridisation. *Chromosoma (Berl.)* **38**, 313-318 (1972).
- Corneo, G., Ginelli, E., Polli, E.: Isolation of the complementary strands of a human satellite DNA. *J. molec. Biol.* **33**, 331-335 (1968).
- Corneo, G., Ginelli, E., Polli, E.: Repeated sequences in human DNA. *J. molec. Biol.* **48**, 319-327 (1970).
- Corneo, G., Ginelli, E., Polli, E.: Renaturation properties and localisation in heterochromatin of human satellite DNA's. *Biochem. biophys. Acta (Amst.)* **247**, 528-534 (1971).
- Corneo, G., Zardi, L., Polli, E.: Elution of human satellite DNAs on a methylated albumin kieselgur chromatographic column: isolation of satellite DNA IV. *Biochim. biophys. Acta (Amst.)* **269**, 201-204 (1972).
- Craig, A. P., Shaw, M. W.: Polymorphism of human constitutive heterochromatin. *Science* **174**, 702-704 (1971).
- Jones, K. W.: Chromosomal and nuclear location of mouse satellite DNA in individual cells. *Nature (Lond.)* **225**, 912-915 (1970).
- Jones, K. W., Corneo, G.: Location of satellite and homogenous DNA sequences on human chromosomes. *Nature (Lond.) New Biol.* **233**, 268-271 (1971).
- Jones, K. W., Prosser, J., Corneo, G., Ginelli, E., Bobrow, M.: Constitutive heterochromatin in man. *Symp. Medica Hoechst* (in press, 1972).
- Lubs, H. A., Ruddle, F. H.: Applications of quantitative karyotyping to chromosome variation in 4 400 consecutive newborn. In: *Human population cytogenetics* (P. A. Jacobs, W. H. Price and P. Law, eds.), p. 119-142. Edinburgh: University Press 1970a.
- Lubs, H. A., Ruddle, F. H.: Chromosomal abnormalities in the human population: estimation of rates based on New Haven newborn study. *Science* **169**, 495-497 (1970b).
- Lubs, H. A., Ruddle, F. H.: Chromosome polymorphism in American negro and white populations. *Nature (Lond.)* **233**, 134-136 (1971).
- Marmur, J.: A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J. molec. Biol.* **3**, 208-218 (1961).
- Prosser, J., Moar, M., Bobrow, M., Jones, K. W.: Satellite sequences in chimpanzee (*Pan troglodytes*). *Biochim. biophys. Acta (Amst.)* (in press, 1973).
- Saunders, G. F., Hsu, T. C., Getz, M., Simes, E. L., Arrighi, F. E.: Locations of a human satellite DNA in human chromosomes. *Nature (Lond.) New Biol.* **236**, 244-246 (1972).

Received December 7, 1972—January 22, 1973 / Accepted by W. Beermann
Ready for press February 10, 1973

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Printed in Germany
Universitätsdruckerei H. Stürtz AG, Würzburg

Reprint from

Modern Aspects of Cytogenetics: Constitutive Heterochromatin in Man

Symposium Schloß Reinhartshausen/Rhein
June 18th – 20th, 1972

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Satellite DNA, Constitutive Heterochromatin, and Human Evolution

K. W. JONES, J. PROSSER*, G. CORNEO, E. GINELLI**, and M. BOBROW***

Introduction

The DNA of constitutive heterochromatin contains highly repetitive nucleotide sequences many of which comprise families of closely related molecules which may be isolated by virtue of their unique buoyant density on isopycnic gradients. As far as we presently know this type of DNA derived from heterochromatin is not transcribed and its function, along with that of repetitive DNA in general, is not understood. These so-called »satellite« DNA's afford a readily available and pure fraction of DNA whose chromosomal and nuclear location can be mapped by *in situ* hybridization of radioactive complementary RNA (c-RNA) (8, 11). Experiments using *in situ* hybridization (12, 13, 14, 17, 19, 21) have demonstrated that satellite DNA is compactly located, predominantly in constitutive heterochromatin, near the centromeres of chromosomes and, similarly, remains as compact blocks during interphase. At the present time, with the exception of closely related subspecies of *Drosophila* (10), particular satellite DNA's have been found to be species-restricted.

Satellite DNA, it has been suggested (2), arises from an original sequence by relatively rapid reduplication events which have been called *saltatory replications*. Initially, the members of such a new family of molecules are virtually identical. This uniformity allows such DNA to renature very rapidly and completely under appropriate conditions following denaturation. The rate of renaturation is dependent upon the bimolecular collision of sequences which are sufficiently similar to form stable duplexes and is therefore directly proportional to the concentration of such molecules in the reaction space. When reacting molecules are identical they re-form duplexes which are not readily distinguishable from the native form. One way of assessing how well renaturation has proceeded is heat denaturation of the renaturation product and establishing its T_m or melting point (defined as the temperature at which 50% of the molecules are rendered single-stranded). This may then be compared with the T_m of the native molecule. The difference (ΔT_m) is related to the extent

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of non Watson-Crick base pairs (mismatching) present in the renatured product, such that 1.6° lowering of the T_m indicates 1% mismatched base pairs (25). Earlier work suggested a higher value of 1° C = 1.5% (15) but our calculations are based on the later value. This is a measure of the amount of base substitution or nucleotide divergence which has arisen among the originally identical members of a family of molecules, such as a pure satellite fraction. Nucleotide sequence divergence is time-dependent and, although there is no generally accepted value for the rate at which this occurs (15), it is clear that ΔT_m is related to the relative age of a DNA family. Measuring the ΔT_m is therefore a way of looking back in evolutionary time to the period at which a given family of molecules emerged.

By this approach, and assuming that satellite DNA changes at the rate of non-repetitive DNA, it has been roughly estimated that mouse satellite DNA originated about 4 million years ago (15). In all probability, however, satellite sequences will change at a faster rate than »unique copy« DNA since the latter presumably contains coding sequences, which will not tolerate certain changes. Estimated values of satellite DNA age based on rates for unique copy DNA are thus in all probability too great by a factor of at least 3-fold, assuming that most nucleotide changes in »unique« DNA concern the codon third base, and that all unique copy DNA is coding.

Human DNA contains three well authenticated satellites (4, 6, 7); all of which have been shown to be associated with heterochromatin, whose relative age may be estimated in the above manner. These estimates provide instructive insights into the origins of human satellite DNA, the course of primate evolution, and the nature of human constitutive heterochromatin. Much of the data which will be presented is as yet unpublished, and in preparation. The senior author therefore takes responsibility for the approach presented and for the interpretations afforded here.

Table 1. Physical properties of human satellite DNA's*

| | Satellite DNA | | |
|-------------------------------|---------------|-------|-------|
| | I | II | III |
| Buoyant density | 1.687 | 1.693 | 1.696 |
| T_m Native | 80° C | 87° C | 84° C |
| T_m Renatured | 74° C | 85° C | 74° C |
| ΔT_m ** | 6° C | 2° C | 10° C |
| % Divergence (1.6° C = 1%) | 3.7% | 1.2% | 6.2% |

* Data adapted from CORNEO et al. (5, 6, 7).
** Uncorrected for single strand hyperchromicity.

Using the factor $1.6^{\circ}\text{C} = 1\%$ base pair mismatching (25) it can be seen from Table 1 that there are considerable differences in the extent of the divergence of the three human satellites. These indicate that their order, in progressively greater age, is II, I, and III.

Given a value for the rate of base substitution in satellite DNA we could arrive at some estimate of the absolute ages represented by these divergences. However no such figure exists, but we can gain a rough maximum estimate by using the values derived for the divergences of single copy DNA during primate evolution. These have been worked out by KOHNE (15) using the value 1°C lowering of the $T_m = 1.5\%$ base pair mismatching, and we have readapted his values for various primate DNA's using the more recently derived value of ULLMAN and MCCARTHY (25) ($1.6^{\circ}\text{C} = 1\%$ mismatching) to allow comparison with Table 1. These are set out in Table 2.

Table 2.

| DNA's compared | Changes since divergence (%)* | Total divergence time (m. yr.)** | % Divergence human sat. DNA |
|--------------------|-------------------------------|----------------------------------|-----------------------------|
| Man - Chimpanzee | 1.00 | 30 | |
| Man - Gibbon | 2.48 | 60 | 1.2 (II) |
| Man - Green Monkey | 4.10 | 90 | 3.7 (I) |
| Man - Rhesus | 4.00 | 90 | |
| Man - Capuchin | 6.96 | 130 | 6.2 (III) |
| Man - Galago | 21.60 | 160 | |

* Adapted from KOHNE (15).
** SIMONS: Scientific American (23).

Comparing Tables 1 and 2, (Column 4 in Table 2) shows that satellite III DNA has diverged from its original sequence almost to the same extent as human DNA has from DNA of capuchin with a paleontologically estimated divergence time of 130 million years (see Fig. 4). About 100 million years then represent a maximum value for the age of human satellite III. If we suppose that the actual rate of divergence of a satellite sequence is about 3-4 times faster, as discussed above, we arrive at a time of 25-30 million years for the age of this satellite (Oligocene). Similar calculations give an estimated age of 20-25 million years for satellite I (Early Miocene) and 9-12 million years (Pliocene) for satellite II. According to current views, chimpanzee and man diverged anywhere between 4 (26) and 30 (Refs. 1, 3, 16, 20, 22, and 24) million years ago. Methods

based upon amino acid sequencing in selected proteins have supported the more recent time (27). According to the above calculations human satellites III, I and II would all predate the latest divergence time of 4 million years. If we use the earlier date, approaching 30 million years, satellite III would be contemporaneous with the date of divergence whilst I and II would have arisen later. In this context it is now interesting to reflect that the unique copy DNA of man and chimpanzee (Table 2) has diverged by only about 1% [using KOHNE (15), but $1.6^\circ \text{C } T_m = 1\%$] so that, unless we suppose that satellite DNA diverges at more than 6 times the rate of unique copy DNA, satellite III must have originated before man and chimpanzee diverged. In this event the same sequence might be present in the chimpanzee. We have established the fact that human satellite III is located in centromeric heterochromatin. A related sequence in the chimpanzee would therefore probably also be visible *in situ*. Accordingly we have hybridized human satellite III with chimpanzee chromosomes. The result is shown in Plate IV where it can be seen that grains lie over the centromeric heterochromatin. It is therefore strongly suggested that our ideas about the relative time of origin of this sequence are reasonable. It may also be concluded that in primates satellite DNA is not species-restricted. Comparison of Plates I and IV show that there are more prominent locations containing satellite III on chimpanzee than on human chromosomes. The inference from these observations is that the chimpanzee must contain an isolatable satellite DNA possibly in relatively greater amount than human satellite III, with identical or very similar properties to human III. To examine this point DNA was obtained from a chimpanzee liver at autopsy shortly after death and analysed for the presence of such a component. Fig. 1a shows the overloaded $\text{Ag}^+ - \text{Cs}_2\text{SO}_4$ gradient profile in which a satellite component may be clearly seen (PROSSER, MOAR, BOBROW and JONES; submitted for publication). This component separates into two satellites on CsCl gradients (Fig. 1b) which we have called A and B. Component A is similar to human satellite III and, as predicted from *in situ* hybridization, is present to a relatively greater amount than is human III in human DNA. It has been isolated and purified and its physical characteristics are compared with those of human satellite III in Table 3.

It is clear from Table 3 that the two satellite components are extremely similar. Chimpanzee satellite A also separates into its component strands, when denatured in an alkaline CsCl gradient, in the same way as does human satellite III DNA. Of particular interest is the fact that the ΔT_m 's of the two DNA's are very alike, differing by only one degree. This argues very strongly in favour of a common time of origin as would be expected if the sequences are closely related and as hybridization *in situ* suggests.

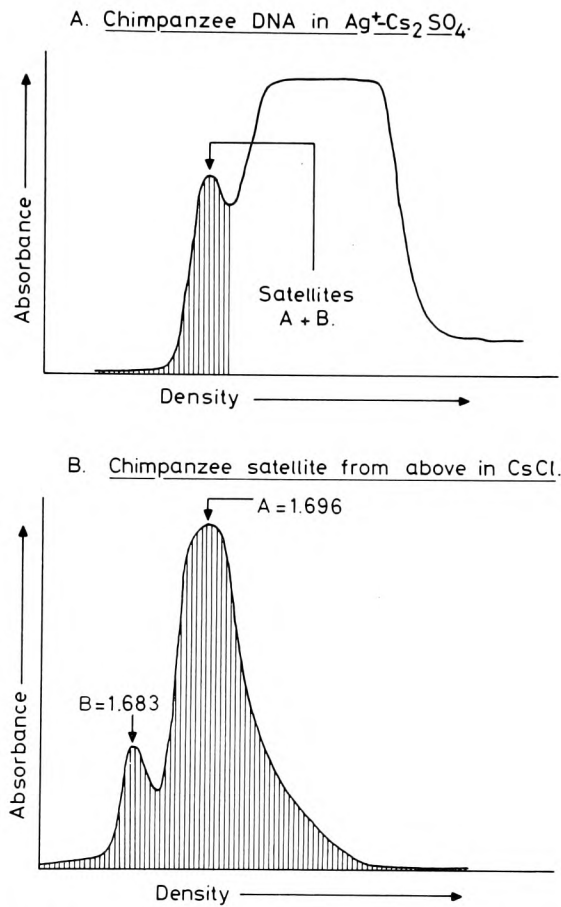


Fig. 1. Separation of chimpanzee satellites A and B on isopycnic gradients. a) Overloaded silver cesium sulfate gradient showing a satellite band on the light side of the main DNA band. This was isolated and re-run in cesium chloride. b) Material isolated from (a) and rebanded on cesium chloride. The original monodisperse satellite peak has separated into two buoyant density components: satellite A, 1.696 g/cm^3 , and satellite B, 1.683 g/cm^3 . Satellite A is the chimpanzee satellite DNA which is homologous with human satellite III and used in the experiments described.

To check further the cross complementarity of chimpanzee satellite DNA (A) with human DNA III we have transcribed it and reacted the radioactive c-RNA with human chromosomes (Plate II). It appears that it cross-reacts with identical heterochromatic regions as those which bind human III. Reacted against its own chromosomes it gives a generally similar, but more complex, pattern than human DNA III which we have not yet examined in detail (Plate III).

Plates I-VII: All plates approximately same magnification; c-RNA to human satellite III approximately 1.1×10^8 DPM/ μ g; c-RNA to chimpanzee satellite A approximately 1.11×10^8 DPM/ μ g. Hybridization carried out in $2-4 \times$ SSC for 6-8 h. Autoradiographic exposure time 4-6 weeks. Ilford K 2 emulsion.

Plate I. Location of human satellite III by *in situ* hybridization of c-RNA on human chromosomes (male). Two chromosomes are mainly involved, which may be pair number 9. Other chromosomes are also involved with minor amount of this sequence but analysis of the overall distribution has not yet been completed (JONES, CORNEO, GINELLI and PROSSER: Chromosoma (Berl.): In press.)

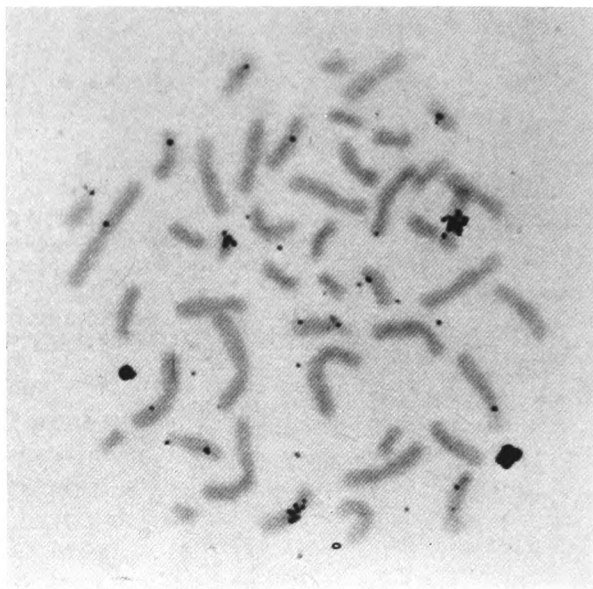
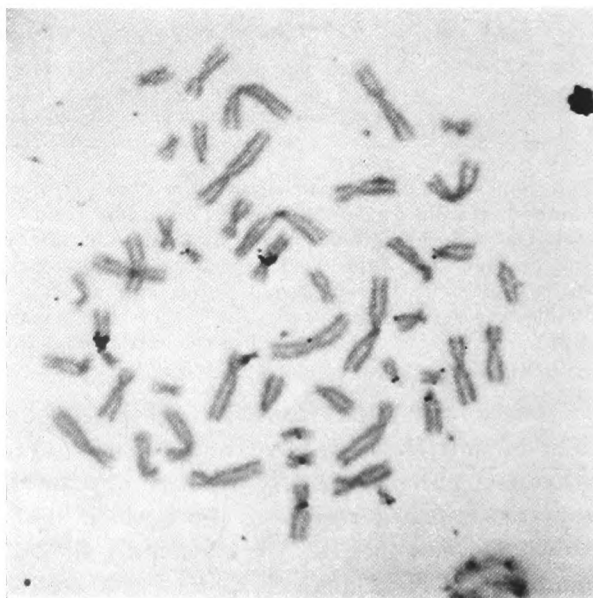


Plate II. Location of satellite sequences homologous with chimpanzee satellite A *in situ* on human female chromosomes. The locations appear to be identical with those of human satellite III shown in Fig. 1. As in the case of human III, other minor locations of the sequence appear in longer exposures but have not yet been examined in detail.



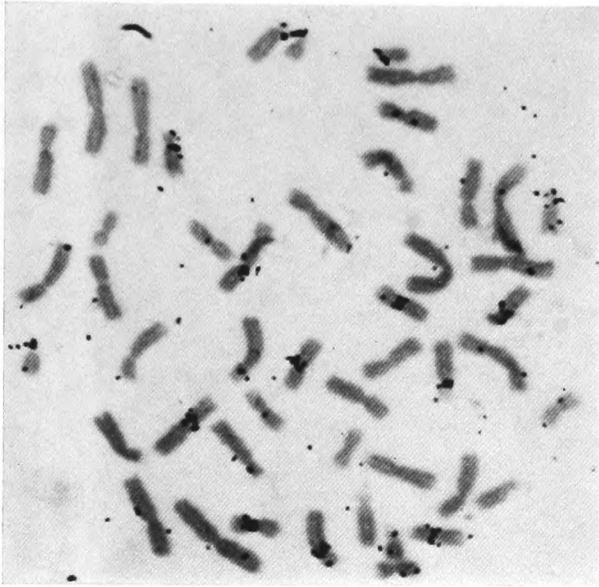


Plate III. Distribution of locations containing chimpanzee satellite A DNA sequences on chimpanzee (female) chromosomes. The satellite c-RNA has mainly hybridized to metacentric chromosomes. Many chromosomes do not possess the sequence in amounts detectable at this exposure time.

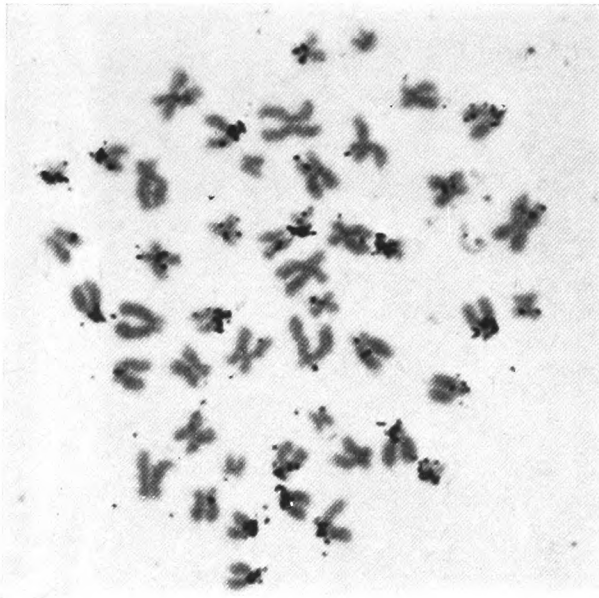


Plate IV. Distribution of sequences complementary to human satellite III on chromosomes of the female chimpanzee. Locations are very similar to that shown in Fig. 3. Complete analysis in preparation.

ERRATUM: The photographs of plate VI and IV have been confused by the printer. The legend of plate VI refers to the photograph of plate IV and vice versa.

Plate V. Location of DNA complementary to chimpanzee satellite A on chromosomes of the female orang-outang. Locations mainly telocentric.

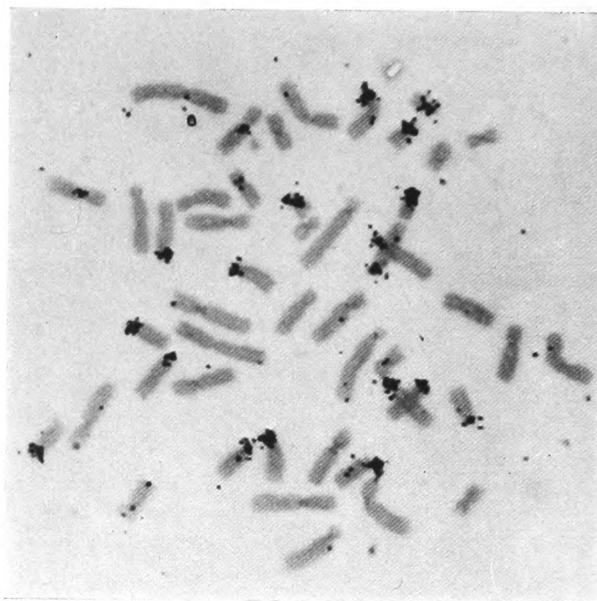
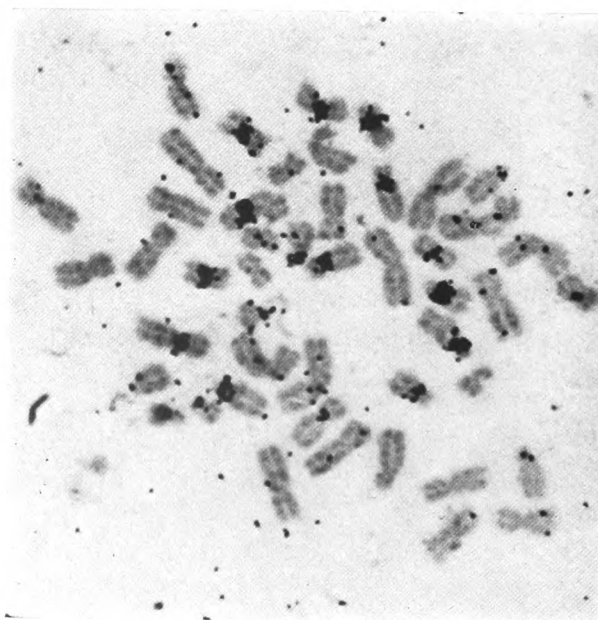


Plate VI. Location of sequences complementary to human satellite III on female orang-outang chromosomes. As in Plate V, these are mainly on telocentric chromosomes.



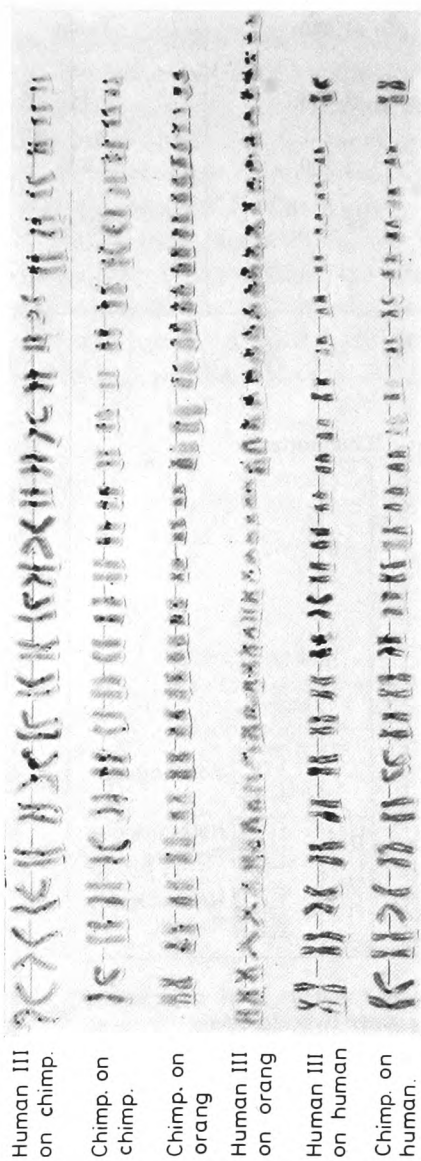


Plate VII. This shows a comparison of the *in situ* hybridization patterns obtained in the various combinations of human III and chimpanzee A satellite c-RNA's with chromosomes of the different species examined. These karyotypes are arranged very approximately according to chromosome size and position of the centromeres with the karyotypes to be compared placed in the same order. The arrangement shown is not claimed to be precise since the data are at present preliminary, however it is evident on inspection that similar chromosomes and groups of chromosomes are affected when comparing chromosomes of a species reacted with either chimpanzee A or human III c-RNA's. Some variation in the extent of hybridization is likely to be due to slight differences in the autoradiographic exposure times.

Table 3

| | Chimp. sat. A | Human sat. III* |
|--------------------------|---------------|-----------------|
| Native | 1.696 | 1.696 |
| Buoyant | | |
| Denaturated | 1.713 | 1.715 |
| Density | | |
| Renaturated | 1.706 | 1.703 |
| T _m (0.1 SSC) | 69° C | 69° C |
| Δ T _m | 9° C | 10° C |

* Adapted from CORNEO et al., 7.

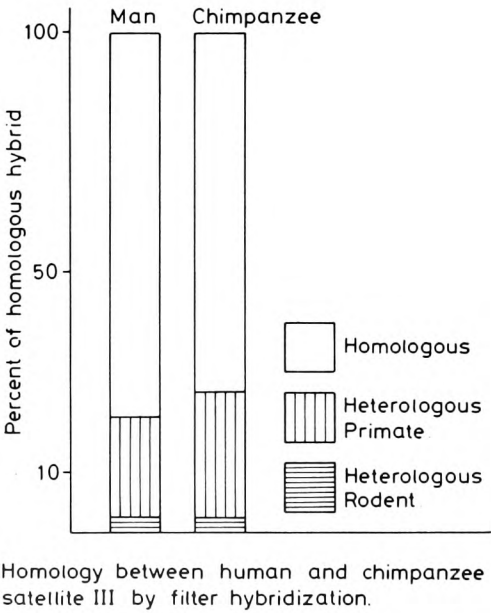


Fig. 2. Results of an experiment in which c-RNA's to human satellite III and chimpanzee satellite A were hybridized for 16 h at 65° C in 2 × SSC with filters loaded with unfractionated human and chimpanzee DNA's respectively and then treated with RNase (20 μg/ml 30 min 37° C in 2 × SSC). The hybridization values obtained in the homologous combinations were very similar and are expressed as 100%. The extent of cross homology is shown as a percentage of this (heterologous primate). The c-RNA's were also reacted with filters loaded with similar amounts of mouse DNA (heterologous rodent) and the values obtained for % hybridization are shown as a fraction of the homologous value.

Filter hybridization of the c-RNA's to human DNA III and chimpanzee satellite DNA (A) has been carried out to check the extent of the homology, and to characterize the hybrid products by thermal elution from filters. The data are presented in Figs. 2 and 3. From Fig. 2 it may be seen that about 22–28% homology still exists between the two sequences and that the thermal elution profiles of the homologous and heterologous reactions are not markedly different (Fig. 3). In the heterologous combination human c-RNA Sat. III reacts to give less stable hybrids with chimpanzee DNA than does chimpanzee satellite A c-RNA with human DNA. The human c-RNA/chimpanzee DNA combination is less stable than the homologous human/human fraction (dotted lines, Fig. 3). This may indicate that the cross-reacting sequences are not identical. Compared with the T_m of the reassociated homologous DNA's (Table 1), the homologous RNA/DNA hybrids have low T_m 's. The reason for this is not clear but it is possible that the RNA polymerase used to transcribe

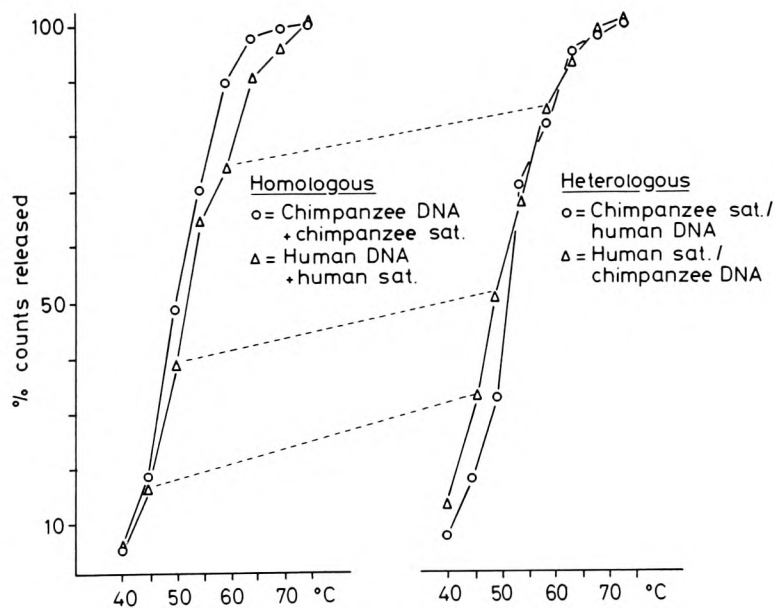


Fig. 3. Filter hybrids formed in the experiments shown in Fig. 2 were disrupted by heating in DSC over a range of increasing temperature. The thermal stability profiles measured as released radioactivity are shown. Dotted lines connect similar temperature points in respect of human homologous and heterologous hybrids and from the slope of these it is apparent that the homologous hybrids are more stable than the heterologous hybrids.

The same tendency is not so evident in the case of chimpanzee hybrids.

the DNA's is selectively copying the A-T rich regions of the template. This needs further study. Mouse DNA does not react appreciably with satellite III or chimpanzee satellite A, and mouse chromosomes do not show any localization *in situ* of either chimpanzee satellite A or human satellite III c-RNA.

Is satellite III present in other primate species ?

The presence of common satellite sequences on chimpanzee and human chromosomes shows that they possessed a common progenitor. The time at which this creature existed will be determined when we understand more accurately how satellite DNA diverges. Towards understanding this aspect we are studying the possible presence of human satellite DNA II and I in chimpanzee since the different ages of these sequences should yield valuable comparative information on the time of origin of man and chimpanzee from a common ancestor, assuming that these satellites behave in a similar way to satellite III. An interesting question is whether or not other primates have satellite III (or chimpanzee satellite A. We have made a start to answering this question by hybridizing c-RNA of satellite III and chimpanzee satellite A to chromosomes of the orang-outang. The results indicate that the same sequence is present and may be detected by hybridizing either c-RNA. As may be seen in Plate V and VI it is chiefly the acrocentric chromosomes of this species which react. The results of all of the *in situ* experiments is shown for comparison in Plate VII, from which it is clear that the groups of chromosomes reacting from any of the 3 species are strikingly similar with either c-RNA. However, there are some indications of difference in detail which we are now analysing; for this reason the identification of chromosomes in Plate VII are tentative. The results indicate that a buoyant density component very similar to chimpanzee A and human III satellites will be discovered in the orang-outang when material becomes available which will provide DNA for analysis.

Do all Primates possess satellite III and chimpanzee (A) sequences ?

So far, satellite III, and chimpanzee satellite A, has been found in all three of the higher Primates examined. The question of whether it is a generalised primate sequence therefore arises. The ages calculated for satellites III and A of 25–30 million years indicate a common origin in the Oligocene. According to tentative paleontological dating, the Cercopithecoidea diverged from the lines leading to Hominidae (man) and Pongidae (chimpanzee, orang-outang, and gorilla) during the Eocene (36 ± 2 million years) (Fig. 4; SIMONS, 23). It is therefore not likely that satellites III and A will be present in members

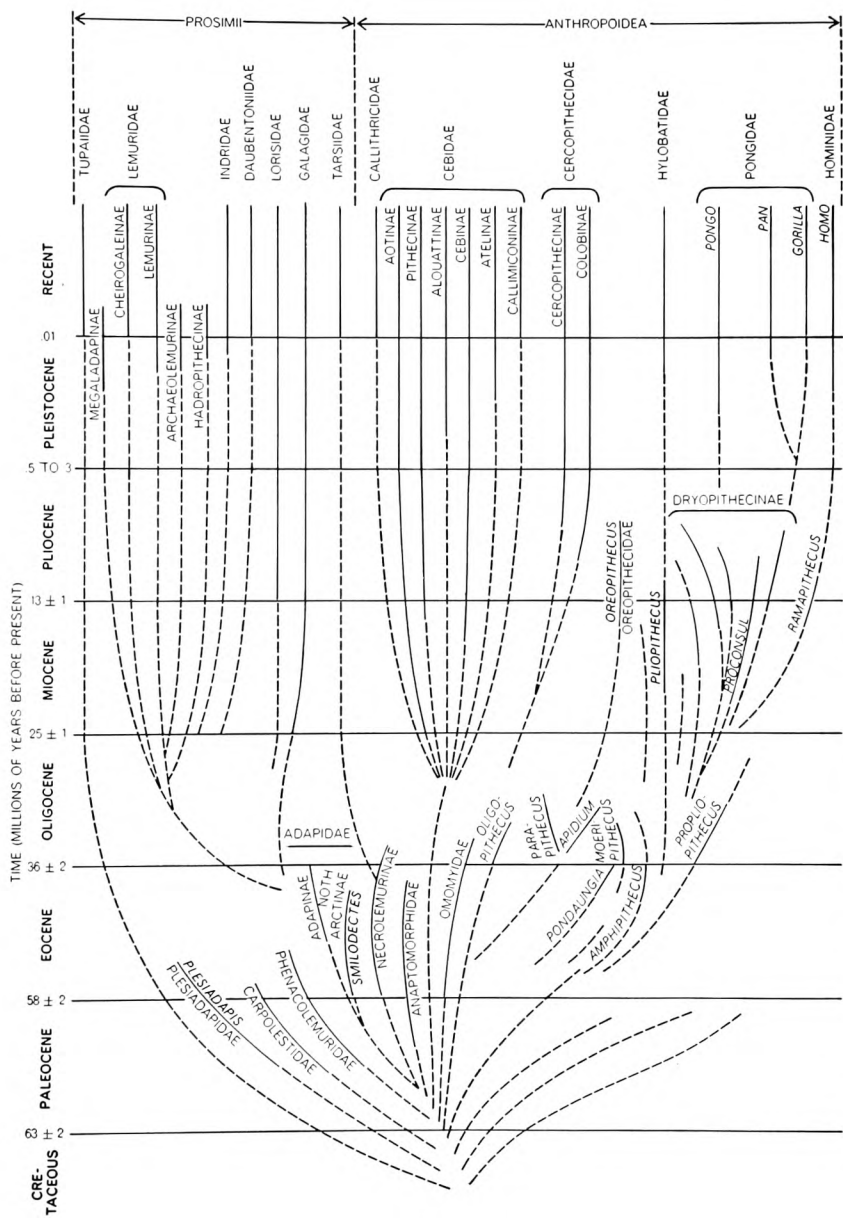


Fig. 4. Phylogeny of the primates. Broken lines show hypothetical relations. Solid lines show the periods when species of the groups named are known to have flourished. (From Simons, E. L.: The Early Relatives of Man. Copyright 1964 by Scientific American, Inc. All rights reserved.)

either of the Cercopithecoidae, or of the Cebidae which form the groups of New World monkeys, if the present estimates of its age are correct. So far we have examined only one species representing each of these groups, the capuchin monkey (Cebidae), and the baboon (Cercopithecoidae), by *in situ* hybridization of chimpanzee satellite A c-RNA. The results have been negative following exposure times which reveal the presence of these sequence in the higher apes. It is too early to conclude finally that the sequence is absent in these groups, but at present the indications are that it may well be. This negative finding supports the present maximum estimates of the age of the sequences and suggests that it can not be more than 25–35 million years old assuming that it has not just been lost from these latter evolutionary lines. It will be of great interest to examine the gibbon in this respect since it is thought to have diverged separately from the common progenitor of the Pongidae and Hominidae at about 25–30 million years.

Conclusions

It is tempting to conclude that the presence of common centromeric DNA sequences among the higher apes and man signifies the existence of an evolutionary mechanism which has potentiated the comparative success of this group in evolution. Until we know more about the role of DNA satellites however it is difficult to suggest their precise role in this context. It does nevertheless appear that compared with other groups, such as rodents, primates have conserved certain DNA families within their constitutive heterochromatin. From this fact it would seem to follow that any benefits conferred by the possession of such conservative sequences will be operational over the long term.

One of the functions of centromeric heterochromatin may be to control the process of chromosomal interchange in the evolution of the karyotype. This process constitutes an important mechanism for speciation. In this regard it is clear that the chromosomes in certain groups of animals are rather more inclined to undergo structural rearrangements than are others. A particularly intriguing example of this is found in certain Teleost fishes, notably *Salmo irideus*, which exhibit epigenetic Robertsonian type fusions so that each tissue comes to possess a more or less characteristic karyotype. It has been suggested (18) that the purpose of this is to bring about changes in the genetic function of the chromosome arms, presumably connected with the physiological functioning of the tissues concerned. In our view, similar chromosomal associations may occur in many other animals through the medium of temporary chromocentre formation in interphase rather than by more permanent fusions. In the mouse (*Mus musculus*) it is clear from *in situ* hybridization, as has been pointed out previously (12, 13, 14), that the chromocentral heterochromatin is

composed of centromeric heterochromatin. This implies that some, at least, of the telocentric mouse chromosomes are, physiologically speaking, temporarily metacentric. It is tempting to speculate that the permanent metacentric arrangements seen, for example, in the tobacco mouse (*M. poschiavinus*) (9) have arisen out of previous physiologically successful, but more tentative, associations occurring in *M. musculus*. Regarded in this way, temporary association of chromosome arms in constitutive heterochromatic chromocentres may constitute a mechanism which allows the testing of potential combinations before committing the organism to the reproductive isolation which tends to accompany more permanent rearrangements.

As the example of *Salmo irideus* so vividly illustrates, the potential to form new chromosomes by centric fusion is possessed to differing degrees by different organisms and by different chromosomes within the same organism (for example telocentrics). How this process is controlled is of first importance for evolution, successful or otherwise. Ultimately, these tendencies must be understood in chemical terms, probably in terms of the chemistry of constitutive heterochromatin and possibly in terms of repetitive DNA sequences. When we reach this stage in our understanding, the significance of common satellite DNA sequences in hominid evolution will perhaps become apparent.

Summary

Constitutive heterochromatin in man contains at least three well authenticated satellite DNA's called satellites I, II, and III. From their thermal stabilities after reassociation it appears that these components are of different ages and therefore have arisen at different periods during human evolution. In this paper we show the chromosomal location of satellite III by *in situ* hybridization of radioactive complementary RNA. The suggested age of satellite III places its origin at a time before the divergence of man and chimpanzee. In confirmation of this, we show that the same sequence is present on chimpanzee chromosomes, and that a satellite sequence, which we have called chimpanzee satellite A may be isolated from chimpanzee DNA. This satellite has virtually identical physical properties to human satellite III and its renaturation properties indicate a similar age. c-RNA to this satellite A hybridizes *in situ* with the same human chromosomes as does human satellite III and similar groups of chromosomes react with either satellite c-RNA in the chimpanzee. Tested against the orang-outang, the satellite c-RNA's react with the same group of acrocentric chromosomes. We conclude from these facts that man, chimpanzee and orang-outang shared a common progenitor. We also conclude that primate satellite sequences are not species-restricted. Satellites III and A do not

apparently hybridize with the baboon or with the capuchin monkey which is in keeping with a paleontologically derived earlier divergence date for these lines of evolution, and which allows us to suggest limits between which the satellite sequences III and A arose in evolution.

Zusammenfassung

Das konstitutive Heterochromatin des Menschen enthält mindestens 3 gesicherte Satelliten-DNS-Fraktionen, die als Satellit I, II und III bezeichnet wurden. Geht man von der Hitzestabilität nach Reassoziationsausgang aus, dann scheinen diese Komponenten verschieden alt und in verschiedenen Perioden der menschlichen Evolution entstanden zu sein. In dieser Arbeit soll die Lokalisation von Satellit I mittels In-situ-Hybridisierung von radioaktiv komplementärer RNS gezeigt werden. Satellit I, so wird angenommen, entstand vor dem Zeitpunkt der Trennung von Mensch und Schimpanse. Zur Bestätigung dieser Hypothese wird die gleiche Sequenz in Schimpansen-Chromosomen nachgewiesen. Die als Schimpansen-Satellit A bezeichnete, aus der Schimpansen-DNS gewonnene Fraktion hat tatsächlich die gleichen physikalischen Eigenschaften wie Menschen-Satellit III, und sein Renaturationsverhalten weist auf ein gleiches Alter hin. RNS, die diesem Satellit A komplementär ist, hybridisiert in situ mit den gleichen menschlichen Chromosomen wie Menschen-Satellit III, und ähnliche Chromosomengruppen reagieren beim Schimpansen mit der dem Satelliten komplementären RNS. Am Orang-Utan getestet, reagieren die dem Satelliten komplementären RNS mit der gleichen Gruppe von metazentrischen Chromosomen. Von diesen Befunden leiten wir für den Menschen, den Schimpansen und den Orang-Utan einen gemeinsamen Vorfahren ab. Satelliten-Sequenzen von Primaten sind demnach nicht artspezifisch. Satellit III und Satellit A hybridisieren offensichtlich nicht mit dem Pavian oder mit dem Kapuzineräffchen, die zu einem paläontologisch früheren Zeitpunkt von dieser Entwicklungsrichtung abgezweigt wurden. Damit zeichnen sich die Grenzen ab, innerhalb derer die Satelliten-Sequenzen III und A während der Evolution entstanden sind.

Abbreviations used: SSC = 0.15 M NaCl, 0.015 M $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$; 2 H_2O ; pH 7.2.
DSC = 0.1 SSC.

References

- (1) BUETTNER-JANUSCH, J.: *Origins of Man*. John Wiley, New York 1966.
- (2) BRITTEN, R. J., D. E. KOHNE: *Science* 161: 529 (1968).
- (3) CAMPBELL, B.: *Human Evolution*. Aldine, Chicago 1966.
- (4) CORNEO, G., E. GINELLI, E. POLLI: *J. molec. Biol.* 23: 619 (1967).

- (5) CORNEO, G., E. GINELLI, E. POLLI: *J. molec. Biol.* 33: 331 (1968).
- (6) CORNEO, G., E. GINELLI, E. POLLI: *J. molec. Biol.* 48: 319 (1970).
- (7) CORNEO, G., E. GINELLI, E. POLLI: *Biochim. Biophys. Acta* 247: 528 (1971).
- (8) GALL, J. G., M. L. PARDUE: *Proc. Nat. Acad. Sci.* 63: 378 (1969).
- (9) GROPP, A., U. TETTENBORN, E. LEHMANN: *Cytogenetics* 9: 9 (1970).
- (10) HENNIG, W., I. HENNIG, H. STEIN: *Chromosoma (Berl.)* 32: 31 (1970).
- (11) JOHN, H. A., M. L. BIRNSTIEL, K. W. JONES: *Nature* 223: 582 (1969).
- (12) JONES, K. W.: *Nature* 225: 912 (1970).
- (13) JONES, K. W., F. W. ROBERTSON: *Chromosoma (Berl.)* 31: 331 (1970).
- (14) JONES, K. W., G. CORNEO: *Nature New Biol.* 233: 268 (1971).
- (15) KOHNE, D. E.: *Quart. Revs. Biophys.* 3: 327 (1970).
- (16) LEAKEY, L. S. B.: *Nature* 217: 827 (1968).
- (17) MACGREGOR, H. C., J. KEZER: *Chromosoma (Berl.)* 33: 167 (1971).
- (18) OHNO, S., C. STENIUS, E. FAISST, M. T. ZENZES: *Cytogenetics* 4: 117 (1965).
- (19) PARDUE, M. L., J. G. GALL: *Science* 168: 1356 (1970).
- (20) PILBEAM, D. R.: *Nature* 219: 1335 (1968).
- (21) SAUNDERS, G. F., T. C., HSU, M. J. GETZ, E. L. SIMES, F. E. ARRIGHI: *Nature New Biol.* 236: 244 (1972).
- (22) SCHULTZ, A. H.: *Yerkes Newsletter* 3: 15 (1966).
- (23) SIMONS, E. L.: *Scientific American* 211: 50 (1964).
- (24) SIMONS, E. L., D. R. PILBEAM: *Folia Primatol.* 3: 81 (1965).
- (25) ULLMAN, J. S., B. J. MCCARTHY: Cited from Brookhaven Symposium, 1971 (In preparation).
- (26) WASHBURN, S. L. in: *Proc. Roy. Anthropol. Inst.* 1967: 21.
- (27) WILSON, A. C., V. M. SARICH: *Proc. Nat. Acad. Sci.* 63: 1088 (1969).